REMARKS

Applicant respectfully requests the Examiner to reconsider the present application in view of the foregoing amendments to the claims and the following remarks.

Status of the Claims

Claims 1, 3, 4, and 6-11 are currently pending in the present application. The Office Action is non-final. Claims 1 and 6 have been amended and claims 2 and 5 have been cancelled without prejudice or disclaimer. No new matter has been added by way of the amendment. For instance, claim 1 has been amended to include textual subject matter taken from claims 2 and 5, now cancelled. Claim 6 was amended to correct typographical errors and to amend its dependency to claim 1. Thus no new matter has been added.

Based upon the above considerations, entry of the present Amendment is respectfully requested.

Claim Objection

Claim 6 is objected to due to informalities. Applicant amended claim 6, without prejudice or disclaimer, to correct the typographical errors. Applicant respectfully requests reconsideration and withdrawal of the present objection.

Issues under 35 U.S.C. § 112, Second Paragraph, Indefiniteness

Claims 1-11 stand rejected under 35 U.S.C. § 112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter, which Applicant regards as the invention.

The Examiner asserts that claims 1-11 are vague and indefinite since the phrase "the DNA preparation" has no antecedent basis. The Examiner additionally asserts that claims 1-11 are vague and indefinite because the preamble recites that the method is for determining the concentration of circulating DNA in a plasma sample from a cancer patient, but the claims do not recite an active method step to determine the concentration of circulating DNA in a plasma sample from a cancer patient.

Although Applicant disagrees, in order to further prosecution, Applicant has amended claim 1, without prejudice or disclaimer, to include textual subject matter taken from claims 2 and 5, now cancelled. Additionally, claim 1 was amended to provide antecedent basis for the term DNA preparation.

Applicant respectfully requests reconsideration and subsequent withdrawal of the present rejection.

Issue Under 35* U.S.C. § 103(a), *Obviousness

Claims 1-4, 7-8 and 10-11 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Chang *et al.*, U.S. Patent No. 6,664,046 (hereinafter "Chang") in view of Cook, U.S. Patent No. 7,160,996 (hereinafter "Cook") and Sozzi *et al.*, "*Analysis of Circulating Tumor DNA in Plasma*

at Diagnosis and During Follow-Up of Lung Cancer Patients," Cancer Research, Vol. 61, pp. 4675-4678, (2001) (hereinafter "Sozzi").

The Examiner asserts that Chang discloses a method of quantitation of expression of hTERT mRNA and that the level of hTERT mRNA expression assists in the diagnosis of cancer. The Examiner also asserts that the Chang method involves amplifying a target hTERT mRNA sequence using a pair of primers, amplification is carried out using a DNA polymerase with 5' to 3' exonuclease activity, amplified hTERT mRNA sequence is detected by probe hybridization where the detection probe is labeled with two fluorescent dyes (one of the dyes is capable of quenching the fluorescence of the other dye and that one dye is attached to the 5' end and the other is attached to an internal site).

The Examiner also asserts that quantitation of a sample containing an unknown number of target sequences typically is carried out with reference to a "standard curve" generated from a series of amplifications of samples containing the target sequence in a range of known amounts.

Chang is asserted to differ from the instantly claimed method only in the use of a quencher/fluorophore reporter system. Cook is relied on for teaching fluorescence energy transfer probes to be used in nucleic acid measurements. Sozzi is relied on by the Examiner for teaching the relationship between plasma DNA concentrations and lung cancer. Applicant respectfully traverses.

Graham v. John Deere, 383 U.S. 1, 17, 148 USPQ 459, 467 (1966), has provided the controlling framework for an obviousness analysis. A proper analysis under § 103(a) requires consideration of the four *Graham* factors of: determining the scope and content of the prior art; ascertaining the differences between the prior art and the claims that are at issue; resolving the

level of ordinary skill in the pertinent art; and evaluating any evidence of secondary considerations (e.g., commercial success; unexpected results). 383 U.S. at 17, 148 USPQ at 467. The *Graham* factors reside in favor of the Applicant, including the factor of determining the scope and content of the prior art.

Applicant has amended claim 1, without prejudice or disclaimer, to include the textual subject matter of non-rejected claim 5, now cancelled.

The present invention is directed to a method of determining the concentration of circulating DNA in a plasma sample by quantifying the hTERT gene (*i.e.*, DNA) present.

The present invention is a methodology of quantification of plasma circulating DNA through the amplification of hTERT copy number as a marker. The invention utilizes a quantification of the total amount of circulating DNA in plasma, including both tumor-derived and host-derived DNA, which implies measuring the effects of an interaction of the tumor with its microenvironment. As opposed to the references relied on by the Examiner, which are related to the detection of specific tumor markers, such as the occurrence of mutation and mRNA over expression of cancer-related genes, the method of the present invention does not measure a tumor specific genetic alteration.

As indicated above, the method proposed by the Applicant is a quantification of the total amount of DNA circulating in plasma using hTERT DNA as a target for real time PCR as opposed to the method disclosed by Chang, which is used for the quantification of expression of hTERT mRNA , *i.e.*, a tumor-specific marker.

The quantification of hTERT mRNA is considered a tumor marker since it can be detected in 85% of the tumors, whereas most healthy tissues exhibit little or no telomerase

expression. In contrast to using the tumor marker hTERT mRNA, the inventor has used hTERT genomic amplification as a marker to quantify, by real-time PCR, the total amount of circulating DNA, which will include not only tumor but also normal DNA, possibly released into the circulation as a result of a tumor-host interaction.

Sozzi *et al.* is relied on for teaching that the mean value of the concentration of plasma DNA in lung cancer patients was higher compared to controls, suggesting the use of plasma DNA as a diagnostic tool for cancer. Sozzi *et al.* is the work of the instant inventor and concerns the former technique proposed by the inventor for quantification of plasma DNA concentration via a colorimetric assay of DNA extracted from plasma without any intervening step (DNA dip-sticks). However, the inventor found that the method in Sozzi *et al.* was not accurate and reproducible enough. The instant invention is an assay for cancer detection based on the quantification of the amount of DNA released into plasma as a result of the interaction of the tumor growth with the surrounding tissue of the host.

The concept underlying the claimed invention is related to the amplification of the short, highly degraded DNA fragments circulating in plasma, in order to perform a precise, robust, reproducible quantification of cell-free DNA circulating in plasma by real-time PCR (*i.e.* using an amplification step that was not even contemplated in the former colorimetric assay previously developed by Applicant).

A more accurate level of quantification was achieved by using continuous values (present method) compared to the use of categorical values (former method of Sozzi *et al.*). This allowed a more adequate definition of plasma DNA as a risk factor for developing cancer as witnessed by

the magnitude of the Odd Risk (OR) observed in individuals with plasma DNA levels higher that certain cut-offs (*i.e.*, OR=85 in individuals with levels ≥20ng/ml).

The genomic structure of hundreds of genes was published and available in public databases (such as for example NCBI) including the hTERT gene at the time of this invention.

The inventor chose, among all the genes of the human genome and over the entire hTERT sequence, primers and probes whose amplification by real-time PCR was specific and robust for the detection of the tiny amounts of highly fragmented DNA, such as that circulating in plasma. hTERT amplification was thus selected for the present application because it fulfilled these features, as opposed to other genes or other regions of the hTERT sequence that showed inferior performance. There is no suggestion in the prior art of the features of claim 1. The specific choice of the specific fragment of hTERT gene recited in claim 1 was not obvious and was the result of a large set of experiments using primers and probes for amplification of a number of different genes.

According to the claimed invention, the total amount of circulating DNA is detected by means of real time PCR using hTERT genomic amplification as a marker (page 4, lines 1-7). The total amount of circulating DNA includes both tumor and "normal" (*i.e.*, not associated to tumor) DNA, possibly released in the circulation as a result of the tumor-host interaction.

The detection of high-level copy number of hTERT in plasma DNA of cancer patients does not reflect the presence of tumor DNA because hTERT is not amplified in the respective tumor DNA. It simply indicates a global level of circulating DNA (as measured by hTERT gene amplification) in plasma of cancer patients, which resulted in significantly higher levels than in healthy controls.

That the amount of circulating DNA does not correlate with hTERT tumor DNA was confirmed by experiments carried out by Applicant. In these experiments, hTERT DNA copy number was analyzed in the tumor tissue of 100 lung cancer patients that showed high level of plasma circulating DNA by hTERT real-time PCR with respect to the copy number of a reference gene. Only in few cases (less then 10%) was a low-level amplification of hTERT DNA was found (relative ratio $\geq$ 2) (See Exhibit 1; Sozzi *et al.*, "*Quantification of Free Circulating DNA as a Diagnostic Marker in Lung Cancer*," Journal of Clinical Oncology, Vol. 21, No. 21, pp. 3902-3908, (2003)) with respect to the copy number of a reference gene and found only in few cases (less then 10%) a low-level amplification of hTERT DNA (relative ratio >= 2). Thus the detection of high-level copy number of hTERT in plasma DNA of cancer patients does not reflect the presence of tumor DNA because hTERT is not amplified in the respective tumor DNA.

The low level of hTERT DNA amplification detected in tumor cells is consistent with the observation that the regulatory mechanism of hTERT expression occurs at the transcriptional (mRNA) level, and not at DNA level. In fact no evidence is provided within the literature on genomic (DNA) amplification of hTERT in primary tumors. It follows that, based on the available scientific knowledge, an increase of telomerase activity, as observed in some tumors, can be related to increased hTERT transcription (mRNA), not to hTERT DNA amplification. The claimed method is not aimed at measuring telomerase activity, *i.e.*, the expression of hTERT protein (or mRNA), but the total circulating DNA by hTERT real-time PCR. Thus, there is no relationship between the measurement of hTERT mRNA, as in Chang *et al.*, and hTERT DNA as with the invention.

Applicant respectfully submits that to date, knowledge regarding the release of DNA into the bloodstream, measured by hTERT copy number, has never been proposed as a tumor-specific marker for cancer, in particular for lung cancer. In fact, as noted above, the regulation of hTERT expression occurs mainly at the transcriptional (mRNA) level.

Moreover a hypothetical amplification of hTERT in tumor would not contribute to the amount of circulating DNA in plasma as the fraction of tumor DNA circulating in plasma is significantly lower that the fraction of normal DNA as demonstrated by the much lower number of DNA fragments containing APC mutation (Exhibit 2: Diehl *et al.*, "*Detection and quantification of mutations in the plasma of patients with colorectal tumors*," Proc. Natl. Acad. Sci. USA, Vol. 102, No.45, pp. 16368-73 (2005)) and p53 mutations (Exhibit 3: Andriani *et al.*, "*Detecting lung cancer in plasma with the use of multiple genetic markers*," Int. J. Cancer, Vol. 108, pp. 91-96, (2004)) compared to the wild-type fragments.

In contrast, most of the referenced prior art documents refer to the quantification of the telomerase activity (*i.e.*, the mRNA expression level), as a tumor marker from plasma or serum, based on the observation that, as said above, telomerase activity is increased in tumors whereas most healthy tissues exhibit little or no telomerase activity/expression. In other words, hTERT is used as a determinant for telomerase activity, and a prerequisite for acquisition of telomerase activity is the expression of a functional hTERT protein.

As discussed above, the teaching of the prior art documents is that plasma or serum hTERT RNA is indicative of tumor gene expression levels and therefore may be used as a tumor marker. In contrast, the claimed invention is concerned with the measurement of total DNA from plasma samples, using hTERT DNA as a target for real time PCR.

The content of the present patent application is almost identically reflected in the paper hereby enclosed, which was published in the Journal of Clinical Oncology (Exhibit 1). The results reported in this paper have been further analyzed and commented in an editorial article published in the same journal number, a copy of which is hereby attached (Exhibit 4: "*Editorial: Early Detection of Lung Cancer Using Serum RNA or DNA Markers: Ready for "Prime Time" or for Validation?*" Journal of Clinical Oncology, Vol. 21, No. 21, pp. 3891-3893, (2003)).

The editorialist clearly distinguishes among the different methodologies thus far proposed for tumor detection by serum or plasma analysis. The technique provided by the authors (G. Sozzi is current inventor and Applicant) is indicated as dealing with total DNA measurement, as opposed for instance to methods for measuring gene expression levels using quantitative PCR technique.

Compared to known techniques (the same disclosed in the prior art cited against the present application), the method of the invention "has the best sensitivity and specificity for detecting cases among all the series reported" (See Exhibit 4, page 3891, right-hand column, lines 9-11). Furthermore, the results presented in the Exhibit 1 article by Sozzi *et al.* (and in the patent application) are considered "provocative, with a sensitivity of 78% and a specificity of 95%, at a cutoff of 15 ng/ml" (See Exhibit 4, page 3892, left-hand column last paragraph). The editorialist concludes that "tests for DNA or RNA alterations in plasma have great potential for early detection and follow-up. This study by Sozzi *et al.* is a step forward in developing such a test."

In light of the above, because there is no teaching, disclosure, reason or rationale provided in the above cited references that would allow one of ordinary skill in the art to arrive at the instant invention as claimed, it follows that the same references are incapable of rendering the instant invention obvious under the provisions of 35 USC § 103(a). Based upon the above, and applying the *Graham factors* analysis test, it is submitted that a *prima facie* case of obviousness has not been established. Applicant respectfully requests reconsideration and withdrawal of the present rejection.

Issue Under 35* U.S.C. § 103(a), *Obviousness

Claims 5-6 stand rejected under 35 U.S.C. § 103(a) as unpatentable over Chang in view of Cook and Sozzi, as applied to claims 1-4, 7-8 and 10-11 above, and in further view of Wick *et al.*, "*Genomic Organization and Promoter Characterization of the Gene Encoding the Human Telomerase Reverse Transcriptase (hTERT),*" Gene, Vol. 232, pp. 97-106, (1999) (hereinafter "Wick"), Buck *et al.*, "*Design Strategies and Performances of Custom DNA Sequence Primers,*" BioTechniques, Vol. 27, pp. 528-536, (1999) (hereinafter "Buck"), and the Search Report from the Examiner.

The Examiner asserts the teachings of Chang, Cook and Sozzi as set forth above. The Examiner states that none of the references discloses SEQ ID NO: 1-3 used as primers and a probe for amplifying the fragment of hTERT gene.

The Examiner further states that Wick discloses the complete genomic organization of the hTERT gene, isolated the 5'- and 3' flanking region and that the hTERT gene encompasses more than 37kb and consists of 16 exons. The Examiner asserts that the results provide the basis

for more detailed studies on the regulation of telomerase activity in normal and cancer cells and may lead to the development of new cancer therapies. As asserted by the Examiner, the search report provided by the Examiner indicates that the nucleic acid sequence of the hTERT gene comprises SEQ ID NO: 1-3.

With regards to Buck, the Examiner asserts that Buck discloses strategies of sequencing primer selection and evaluated primer performance in automated DNA sequencing.

The Examiner additionally asserts that it would have been *prima facie* obvious to apply SEQ ID NO: 1-3 as primers and probes for amplifying a fragment of hTERT gene based on the above references. Applicant respectfully traverses.

Applicant has cancelled claim 5, without prejudice or disclaimer, thus obviating the rejection as to this claim. Since Applicant has incorporated the textual subject matter of claim 5 into claim 1, and amended claim 6 to depend from claim 1, Applicant will address the rejection as it pertains to amended claim 1.

The previous discussion from the rejection above is herein incorporated by reference. In addition to the previously discussed differences of the present invention to that of the cited references (which indicate that a *prima facie* case of obviousness was not met), Applicant respectfully submits that the Examiner's reliance on a reference, which discloses the entire hTERT gene sequence, is insufficient in supporting the present rejection. The Examiner notes that the hTERT gene is 37 kb. The nature of this art is highly unpredictable. Applicant submits that the Examiner has failed to support why one skilled in the art would be led to select the specific 98 base sequence of claim 1 from an entire gene sequence consisting of 37,000 bases.

In light of this and the previously discussed arguments, because there is no teaching, disclosure, reason or rationale provided in the above cited references that would allow one of ordinary skill in the art to arrive at the instant invention as claimed, it follows that the same references are incapable of rendering the instant invention obvious under the provisions of 35 USC § 103(a). Based upon the above, and applying the *Graham factors* analysis test, it is submitted that a *prima facie* case of obviousness has not been established. Applicant respectfully requests reconsideration and withdrawal of the present rejection.

Issue Under 35* U.S.C. § 103(a), *Obviousness

Claim 9 stands rejected under 35 U.S.C. § 103(a) as unpatentable over Chang in view of Cook and Sozzi, as applied to claims 1-4, 7-8 and 10-11 above, and in further view of Gocke *et al.*, U.S. Patent No. 6,156,504 (hereinafter "Gocke").

The Examiner asserts the teachings of Chang, Cook and Sozzi as set forth above. The Examiner states that none of the references disclose the limitation of claim 9.

The Examiner does assert that Gocke discloses the methods for detecting the presence of extracellular DNA in blood plasma via DNA amplification for the detection, monitoring or evaluation of cancer or premalignant conditions.

The Examiner further asserts that it would have been *prima facie* obvious to carry out evaluation of the risk of cancer development in smokers based on the above references.

Applicant respectfully traverses.

The previous discussions from the rejections above are herein incorporated by reference.

As a general comment to the objections raised by the Examiner, Applicant wants to further clarify and stress an important conceptual difference between present invention and the cited references Chang and Gocke. As indicated above, Applicant has discussed the differences from the present invention and Chang and here incorporates them by reference.

With regards to Gocke, the reference discloses the detection in plasma or serum of nucleic acids derived from mutant oncogenes (K-RAS) or tumor-associated genes (p53, bcl-2, translocations), thus relate to the detection of specific tumor markers. This is profoundly different from the present invention (as discussed above) that is related to the quantification of the global amount of DNA circulating in plasma (both tumor-derived and host-derived), which therefore takes into account the effects of the interaction between the tumor and its microenvironment.

As indicated in the above discussions relating to the conceptual differences of the present invention to the cited references, because there is no teaching, disclosure, reason or rationale provided in the cited references that would allow one of ordinary skill in the art to arrive at the instant invention as claimed, it follows that the same references are incapable of rendering the instant invention obvious under the provisions of 35 USC § 103(a). Based upon the above, and applying the *Graham factors* analysis test, it is submitted that a *prima facie* case of obviousness has not been established. Applicant respectfully requests reconsideration and withdrawal of the present rejection.

In view of the above remarks, Applicant believes the pending application is in condition for allowance.

CONCLUSION

A full and complete response has been made to all issues as cited in the Office Action. Applicant has taken substantial steps in efforts to advance prosecution of the present application. Thus, Applicant respectfully requests that a timely Notice of Allowance issue for the present case.

In view of the above remarks, it is believed that claims are allowable.

Should there be any outstanding matters that need to be resolved in the present application, the Examiner is respectfully requested to contact MaryAnne Armstrong, Ph.D., Reg. No. 40,069 at the telephone number of the undersigned below, to conduct an interview in an effort to expedite prosecution in connection with the present application.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37.C.F.R. §§1.16 or 1.17; particularly, extension of time fees.

Dated: SEP 2 2008

Respectfully submitted,

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Attachments: Exhibit 1; Sozzi *et al.*, "*Quantification of Free Circulating DNA as a Diagnostic Marker in Lung Cancer*," Journal of Clinical Oncology, Vol. 21, No. 21, pp. 3902-3908, (2003))

Exhibit 2: Diehl *et al.*, "*Detection and quantification of mutations in the plasma of patients with colorectal tumors*," Proc. Natl. Acad. Sci. USA, Vol. 102, No.45, pp. 16368-73 (2005)

Exhibit 3: Andriani *et al.*, "*Detecting lung cancer in plasma with the use of multiple genetic markers*," Int. J. Cancer, Vol. 108, pp. 91-96, (2004)) compared to the wild-type fragments.

Exhibit 4: "*Editorial: Early Detection of Lung Cancer Using Serum RNA or DNA Markers: Ready for "Prime Time" or for Validation?*" Journal of Clinical Oncology, Vol. 21, No. 21, pp. 3891-3893, (2003)).

Quantification of Free Circulating DNA As a Diagnostic Marker in Lung Cancer

By Gabriella Sozzi, Davide Conte, MariaElena Leon, Rosalia Cirinciane, Luca Roz, Cathy Ratcliffe, Elena Roz, Nicola Cirenei, Massimo Bellomi, Giuseppe Pelosi, Marco A. Pierotti, and Uga Pastorino

Purpose: **Analysis of circulating DNA in plosmo can provide a useful marker for eorlier lung cancer detection. This study was designed to assess the sensitivity and specificity of a quantitative molecular assay of circulating DNA to identify patients with lung cancer and monitor their disease.**

Materials and Methods: **The amount of plasma DNA wos determined through the use of real-time quantitative polymerose chain reaction (PCR) amplificotion of the humon telomerase reverse transcriptose gene (*hTERT*) in 100 non-smoll-cell lung cancer potients and 100 age-, sex-, and smoking-matched controls. Screening performonce af the assoy wos colculated through the receiver operating chorocteristic (ROC) curve. Odds rotios were colculoted using conditionol logistic regression onolysis.**

Results: **Medion concentrotion of circulating plosmo DNA in patients was almost eight times the volue detected in controls (24.3 v 3.1 ng/mL). The oreo under the ROC curve was 0.94 (95% CI, 0.907 to 0.973). Plasma DNA was a strong risk factor for lung cancer; concentrations in the upper tertile were associoted with an 85-fold higher risk than were those in the lowest tertile.**

Conclusion: **This study shows that higher levels of free circulating DNA can be detected in potients with lung cancer compared with disease-free heavy smokers by a PCR assoy, and suggests o new, noninvosive opproach for eorly detection of lung cancer. Levels of plosmo DNA could also identify higher-risk individuals for lung cancer screening ond chemoprevention trials.**

J Clin Oncol 21:3902-3908. **© *2003 by Americon Society of Clinical Oncology.***

LUNG CANCER is the leading cause of cancer mortality throughout the world and is the cause of more than 1 million annual deaths.[1] In Europe, of the more than 150,000 new patient cases diagnosed every year, only 10% can be cured and can benefit from long-term survival because of the absence of early detection plans, the frequency of metastases at diagnosis, and poor responsiveness to chemotherapy. However, survival of patients undergoing lung resection for small intrapulmonary cancers is greater than 80%.[2] Despite major potential for prevention, complete eradication of smoking has proven difficult, and the risk of cancer remains high in former smokers. As a consequence, there is a need to develop new tests that may facilitate earlier diagnosis and more effective treatment. Low-dose spiral computed tomography (CT) scan of the chest has been effective in detecting small tumors, with a high proportion of resectable (96%) and stage I (80%) disease.[3] Conversely, increased knowledge of molecular pathogenesis of lung cancer offers a basis for the use of molecular markers in biologic fluids for early detection as well as identification of higher-risk smokers.

Common genetic alterations in lung carcinogenesis include allelic loss and instability at loci on 3p (fragile histidine triad [*FHIT*]), 9p ($p16^{INK4A}$), and 17p ($p53$)[4-7]; aberrant promoter methylation of $p16^{INK4A}$, *APC* (adenomatous polyposis of the colon), and other tumor suppressor genes[8,9]; and Kirsten rat sarcoma (*KRAS*)[10] and *p53* mutations.[11-13] Detection of these changes in DNA derived from body fluids such as sputum, bronchial brush and lavage, and plasma or serum of lung cancer patients and chronic smokers has been proposed by several authors as a potential diagnostic tool.[14-24] However, the sensitivity and specificity of detection assays in these biologic samples have been limited by the low frequency of alterations of each specific gene, relative low-sensitivity of used methodologies, and choice of appropriate markers.

Analysis of circulating DNA in plasma is a promising noninvasive diagnostic tool, requiring only a limited blood sample. The intent of this study was to set up a relatively simple blood test on the basis of a single marker, to be potentially applicable to large-scale trials for early lung cancer detection. In a previous report, using a DNA colorimetric assay, we have shown a higher plasma DNA concentration in 84 lung cancer patients than in 43 controls, regardless of tumor stage, suggesting that plasma DNA was an early event in lung carcinogenesis.[25] In addition, changes in DNA level and in the presence of allelic imbalances at 3p loci correlated with the clinical status of patients during follow-up. These results were recently confirmed in a group of 54 patients with ovarian cancer and 31 controls, for whom plasma DNA concentrations and digital single-nucleotide polymorphism anal-

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Authors' disclosures of potential conflicts of interest are found at the end of this article.

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ysis of allelic imbalances were proposed as screening tools for ovarian cancer.[26]

To measure with greater accuracy the amount of free circulating DNA, a quantification approach based on real-time quantitative polymerase chain reaction (PCR) was developed. Using Epstein-Barr virus DNA as the target genome, real-time PCR has proven effective to monitor the progress of nasopharyngeal cancer and assess the effects of treatment.[27] A single copy gene, the amplification of which is specific and robust, represents the ideal target for DNA-based quantitative real-time PCR assay. For this study, we selected an assay designed for the human telomerase reverse transcriptase (*hTERT*) genomic sequence that performed consistently in preliminary experiments. Amplification of *hTERT* was therefore used as a marker of the total amount of DNA present in plasma samples. We considered that *hTERT* expression and telomerase activity have been reported as prognostic factors in stage I non–small-cell lung cancer (NSCLC) patients.[28] However, our working hypothesis was not based on the evaluation of *hTERT* expression at the transcriptional level as a tumor-associated marker, but was based instead on the use of a single copy gene such as *hTERT* as an indicator of the global amount of circulating DNA.

The sensitivity and the specificity of the test were validated in a large case-control study of 200 individuals and in a group of age-matched individuals who had never smoked.

MATERIALS AND METHODS

Patients and Control Series

We evaluated 100 consecutive patients with NSCLC, 81 men and 19 women, who were not previously treated with chemoradiotherapy and were included in the European Institute of Oncology tissue bank from 2000 to 2001. All patients had primary cancers and were receiving first treatment; no patients with disease relapse or follow-up were included.

One control was selected for each patient, matched by sex, age, and smoking habits. Mean age ± standard deviation was 65.1 ± 8.9 years in patients and 64.1 ± 8.2 years in controls; average smoking duration was 40.5 ± 10.9 years in patients and 41.7 ± 9.5 years in controls. The population included seven case-control pairs of never smokers. The 93 heavy-smoker controls were selected among the participants of the European Institute of Oncology early detection program, whose chest spiral CT scans were negative. This prospective study accrued 1,035 volunteers aged 50 years or older who were current or former smokers with a minimum pack/yr index of 20, to be investigated with low-dose spiral CT every year for 5 years. Nonsmoking controls were recruited from healthy blood donors at the immunohematology unit of the Istituto Nazionale Tumori (Milan, Italy). The number of former smokers differed slightly between patients and controls (28 patients and 11 controls). One light and occasional smoker (< 5 cigarettes/d) was matched to a never smoker. A mean within-pair difference of 1 year in age was statistically significant ($P < .02$), suggesting the need to adjust for age in the analysis of plasma DNA as a risk factor.

Sample Collection and DNA Isolation

A 7.5-mL sample of peripheral blood was collected in tubes containing EDTA, from patients before surgery and from controls at the time of spiral CT examination, and stored at −140°. Plasma separation and DNA extraction were performed as previously reported.[25] The DNA purified from 1 mL of plasma was eluted in a final volume of 50 mL of water. Testing of plasma DNA was performed by technicians with no knowledge of the patient or control status.

DNA Quantification in Plasma

To quantify the circulating DNA in plasma, we used a real-time quantitative PCR approach based on the 5′ nucleotide method. This methodology is based on continuous monitoring of a progressive fluorogenic PCR by an optical system.[29,30] The PCR system uses two amplification primers and an additional amplicon-specific and fluorogenic hybridization probe, the target sequence of which is located within the amplicon. The probe is labeled with two fluorescent dyes. One serves as a reporter on the 5′ end (VIC dye; Applied Biosystems, Foster City, CA). The emission spectrum of the dye is quenched by a second fluorescent dye at the 3′ end (TAMRA; Applied Biosystems). If amplification occurs, the 5′ to 3′ exonuclease activity of the AmpliTaq (Applied Biosystems) DNA polymerase cleaves the reporter from the probe during the extension phase, thus releasing it from the quencher.[31] The resulting increase in fluorescent emission of the reporter dye is monitored during the PCR process.

Primers and probes were designed to specifically amplify the ubiquitous gene of interest, the *hTERT* single copy gene mapped on 5p15.33. The amplicon size of the *hTERT* gene was 98 bp (position 13059 to 13156, GenBank accession number AF128893). The sequences of the primers and of the probe were the following: primer forward, 5′-GGC ACA CGT GGC TTT TCG-3′; primer reverse, 5′-GGT GAA CCT CGT AAG TTT ATG CAA-3′; probe, VIC5′-TCA GGA CGT CGA GTG GAC ACG GTG-3′ TAMRA.

Fluorogenic PCRs were carried out in a reaction volume of 50 mL on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Fluorogenic probe and primers were custom synthesized by Applied Biosystems. Each PCR reaction mixture consisted of 25 μL of TaqMan Universal Master Mix (Applied Biosystems), 0.67 μL of probe (15 nmol/L), 0.45 μL of primer forward (10 nmol/L), 0.45 μL of primer reverse (10 nmol/L), and 18.43 μL of sterile water. DNA solution (5 μL) was used in each real-time PCR reaction. Thermal cycling was initiated with a first denaturation step of 50°C for 2 minutes and then 95°C for 10 minutes. The thermal profile for the PCR was 95°C for 15 seconds and 60°C for 1 minute. Data obtained during 50 cycles of amplification were analyzed.

Amplifications were carried out in 96-well plates in a GeneAmp 5700 Sequence Detection System. Each plate consisted of patient samples in triplicates and multiple water blanks as negative control. For construction of the calibration curve on each plate, we used a standard TaqMan Control Human Genomic DNA (Applied Biosystems) at 10 ng/μL with appropriate serial dilutions at 50, 5, 2.5, and 0.5 ng, and 250, 50, and 10 pg. Linear amplification down to the last dilution point representing 10 pg of target DNA was obtained in each experiment (correlation coefficient, 0.999 to 0.995; slope, 3.25 to 3.35).

All of the data were analyzed using the Sequence Detection System software (Applied Biosystems) to interpolate the standard amplification curve of DNA at a known quantity with amplification cycle threshold of the unknown target sample, thus obtaining the relative amount of DNA in the experimental sample.

For the follow-up study, all of the consecutive plasma samples for each patient were simultaneously analyzed in the same real-time PCR experiment to allow comparative quantification of samples along the observation time.

Pathologic and Immunohistochemical Methods

Clinicopathologic data were available for all patients. There were 58 adenocarcinomas, 34 squamous cell carcinomas, three large-cell carcinomas, three pleomorphic carcinomas, and two adenosquamous carcinomas. According to the WHO classification of lung adenocarcinoma,[32] 19 (32.6%) showed an acinar growth pattern, 19 were papillary, 17 (29.3%) were solid, and three (5.2%) were bronchioloalveolar. According to the revised lung cancer staging system,[33] tumor stage was pT1 in 18%, pT2 in 55%, pT3 in 21%, and pT4 in 6% of patients; 47% of patients were pN0, 20% were pN1, and 33% were pN2 or N3. Pathologic stage distribution was IA in 16, IB in 18, IIB in 25, IIIA in 33, IIIB in five, and IV in three patients. In three patients, the analysis was performed only on mediastinal node metastases.

In every patient, the occurrence of either tumor necrosis or lymphoid infiltrate was evaluated semiquantitatively on a scale from absent to 2+ (1+ if ≤ 50% and 2+ if > 50% of the whole tumor). For immunohistochemical

analysis, formalin-fixed and paraffin-embedded samples obtained during surgery were investigated for cell apoptosis-related (p53) and tumor growth (CD117, Ki67, and microvessel density) markers, according to previously refined methods.[34] All patients were evaluated blindly without knowledge of the patients' identity, pathologic diagnosis, clinical outcome, or plasma DNA status. The percentage of p53+, epidermal growth factor receptor (EGFR)+, CD117+, and Ki67-immunoreactive tumor cells was evaluated by scoring a minimum of 1,000 tumor cells in representative fields of immunostaining. Tumor neoangiogenesis was inferred by CD34 immunostaining of endothelial cells as previously described.[35]

Statistical Methods

The distribution of DNA values revealed a departure from normality that was mitigated using a logarithmic transformation. The log of the concentration was used for testing purposes; however, untransformed values were used for reporting results.

Odds ratios (OR) and corresponding 95% CIs were calculated using conditional logistic regression in SAS software (SAS Inc, Cary, NC) to assess plasma DNA as a risk factor for NSCLC. A receiver operating characteristic curve (ROC) was developed to evaluate the diagnostic performance of plasma DNA concentrations. Each unique DNA value was used as a cutpoint to calculate sensitivity and specificity values defining the curve and the area under the curve (AUC). SEs were estimated separately as described in van der Schouw[36] to provide a 95% CI for the area.

The potential association between the logarithm of plasma DNA and demographic, clinical, and immunohistochemical variables in lung cancer patients was explored by running linear regression models using SAS. First, the logarithm of the plasma DNA was regressed on single independent variables by running nine different models (data not shown). Those variables with a coefficient-associated P value $\leq .2$ in simple regression were selected to be included in the multiple regression model. Comparison of median DNA plasma concentrations in patients with follow-up data was done using the Kruskal-Wallis test.

RESULTS

Quantitative Analysis of Circulating Plasma DNA in Cancer Patients and Controls

Figure 1 shows amplification plots of fluorescence intensity against the PCR cycle from plasma samples of cancer patients and matched controls. Each plot corresponds to the initial target DNA quantity present in the sample. Calculation of the amounts of plasma DNA is based on the cycle number, where fluorescence of each reaction passes the cycle threshold, which is set to the geometric phase of the amplification above the background. The x-axis denotes the cycle number of a quantitative PCR reaction. The y-axis denotes the log of fluorescence intensity over the background (ΔRn). The relative amount of plasma DNA is much higher in patient samples (left plots) compared with those of controls (right plots). The amplification curves that are shifted to the right, representing reduced target DNA quantity, clearly discriminate controls from cancer patients. In Figure 2, the distribution of plasma DNA concentration in patients and matched controls describes two distinct populations of values, despite some overlap. The box is bounded below and above by the 25% and 75% percentiles, the median is the solid line in the box, and the lower and upper error bars indicate 90% of values. Median concentration in patients (24.3 ng/mL) was almost eight times the value detected in controls (3.1 ng/mL). High concentrations were observed only in patients, whereas at the other end of the distribution there were few patients with low concentrations of DNA (ie, 0.5 ng/mL). A greater variability of circulating DNA was observed in patients than in controls (Fig 2).

Plasma DNA Concentration As a Risk Factor for NSCLC

An elevated concentration of circulating plasma DNA was associated with a higher risk of NSCLC. Tertile stratification showed that the risk increased exponentially when study participants with plasma concentrations in the second and third tertile were compared with those in the first tertile using conditional logistic regression (up to 85-fold; Table 1). When analyzed as a continuous distribution, a unit increase in plasma DNA (nanograms per milliliter) was associated with a 21% increase in NSCLC risk (OR, 1.21; 95% CI, 1.11 to 1.31).

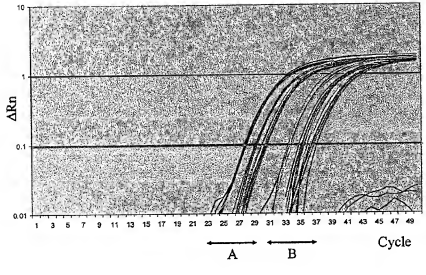

Fig 1. Amplification plots of the real time quantitative polymerase chain reaction for *hTERT* in plasma DNA from (A) non-small-cell lung cancer patients (B) and control samples. The relative amount of plasma DNA is much higher in patient samples (A) compared with controls (B). ΔRn, level of fluorescence detected.

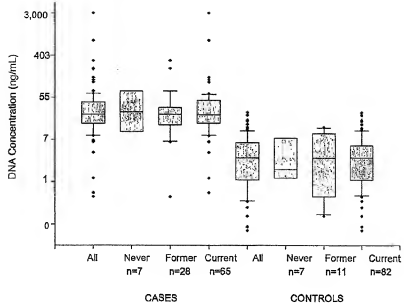

Fig 2. Box-plots of plasma DNA concentration in non-small-cell lung cancer patients and matched controls, according to smoking status. Mini, minimum value detected; max, maximum value detected; sd, standard deviation.

Percentile distribution of plasma DNA concentration in ng/mL by smoking and disease status

	Mini	10^{th}	25^{th}	median	75^{th}	90^{th}	max	mean	sd
Cases	0.5	9.0	15.8	24.3	43.7	65.9	3010	75	310
Never	7.2	7.2	10.7	27.4	74.6	210.7	210.7	59.9	71.3
Former	0.5	6.8	15.0	24.4	33.2	66.3	317.2	41.5	66.7
Current	0.6	9.2	16.1	23.9	45.2	61.4	3010	91	381
Controls	0.1	0.4	1.1	3.1	6.3	11.1	27.0	4.6	5
Never	0.1	0.1	1.2	1.8	7.9	8.4	8.4	.4	3.4
Former	0.2	0.3	0.5	3.1	10.0	11.0	13.5	5.1	5.0
Current	0.1	0.6	1.1	3.1	5.4	11.3	27.0	4.6	5.2

Diagnostic Performance of Real-Time Quantitative PCR Assay

The area under the ROC curve shown in Figure 3 was 0.94 (95% CI, 0.907 to 0.973), suggesting a strong discrimination power of the molecular assay. The curve and AUC were estimated using the logistic procedure in SAS software. Table 2 lists a few of the DNA concentration cutpoints used to generate the curve with their sensitivity, specificity, positive predictive value, and negative predictive value. The 95% CIs around sensitivity estimates overlapped between the successive concentrations shown in Table 2, except for the last two concentrations (20 and 25 ng/mL).

Table 1. Plasma DNA Concentration As a Risk Factor for NSCLC

Tertile Distribution of DNA (ng/mL)*	Patients (n = 100)	Controls (n = 100)	Conditional† Odds Ratio	95% CI
≤ 4	4	62	1‡	
4.1-20	27	36	5.5	1.9 to 16.3
> 20	69	2	85.5	16.5 to 445

Abbreviation: NSCLC, non-small-cell lung cancer.
*Obtained from the pooled distribution of plasma DNA values in cases and in controls.
†Adjusted for age.
‡Reference group.

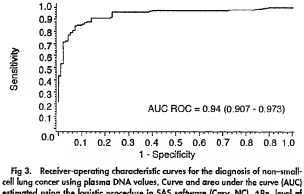

Fig 3. Receiver-operating characteristic curves for the diagnosis of non-small-cell lung cancer using plasma DNA values. Curve and area under the curve (AUC) estimated using the logistic procedure in SAS software (Cary, NC). ΔRn, level of fluorescence detected; ROC, receiver operating characteristic curve.

Table 2. Screening Performance of Plasma DNA Concentration

Cutpoint (ng/mL)*	Sensitivity	95% CI†	Specificity	95% CI†	PPV	NPV
4	97	91.5 to 99.4	60	49.7 to 69.7	71	95
7	92	84.8 to 96.5	77	67.5 to 84.8	80	91
10	88	80.0 to 93.6	86	77.6 to 92.1	88	88
15	78	68.6 to 85.7	95	88.7 to 98.4	94	81
20	69	58.9 to 77.9	98	93.0 to 99.8	97	76
25	46	35.9 to 56.3	99	94.5 to 100	98	65

Abbreviations: PPV, positive predictive value; NPV, negative predictive value.
*Contrasting concentrations lower than the cutpoint against concentrations equal to or greater than the value specified.
†Sensitivity and specificity estimates were treated as binomial parameters to calculate 95% CIs using Statxact (Cambridge, MA).

Correlation of Plasma DNA Levels With Clinicopathologic Features

Plasma DNA was significantly associated with age, and increased with increasing age after adjusting for remaining variables in the model (Table 3). No association was observed between plasma DNA levels and smoking intensity or duration, cell type, pathologic stage, or other features such as necrosis, lymphoid infiltration, or growth patterns.

The association between plasma DNA and microvessel density was modified by the age of the patient (significant interaction): plasma DNA increased with increasing microvessel density in younger and not in older patients. Ki67 and EGFR expression were not statistically associated with plasma DNA after controlling for differences in the other variables in the model. Addition of number of cigarettes smoked did not introduce any relevant change in the model. Regression of all variables explained 29% of the observed variability in plasma DNA.

Change in Plasma DNA Levels During Follow-Up

In 35 cancer patients, a second plasma sample was collected, 3 to 15 months after surgery (median elapsed time, 8 months), and analyzed to monitor changes in DNA levels during clinical follow-up. The overall median DNA concentration in follow-up plasma samples was 8.4 ng/mL, showing a clear trend toward reduction, compared with median baseline levels of 24.5 ng/mL ($P < .0001$). When these patients were tested according to their clinical status, median DNA concentration at follow-up was significantly lower in 30 disease-free individuals as compared with the five cancer patients with proven cancer relapse (7.1 *v* 24.7 ng/mL; $P = .002$). Figure 4 shows the reduction in DNA levels of each patient, stratified by relapse status.

Table 3. Correlation of Plasma DNA Concentration With Clinical and Pathologic Parameters: Multiple Linear Regression of the Logarithm of Plasma DNA (ng/mL)

Variable	Coefficient	SE	P
Intercept	3.429	0.974	.001
Age, years			
≤ 60 (1)	−6.262	1.682	.0003
61-71 (2)	−2.280	1.198	.06
≥ 72 (3)	Reference		
Log mean MVD, continuous distribution	0.0001	0.262	.99
Log mean MVD × age (1)	1.596	0.469	.001
Log mean MVD × age (2)	0.475	0.346	.17
EGFR			
< 70%	0.379	0.227	.09
≥ 70%			
Ki-67 %, continuous distribution	0.007	0.005	.17
Smoking, No. of cigarettes/d	−0.009	0.006	.15

NOTE. Numbers in parentheses (1, 2, and 3) represent the three age classes used for linear regression analysis of log mean MVD, as presented in this Table.
Abbreviations: MVD, microvessel density; EGFR, epidermal growth factor receptor.

DISCUSSION

Previous studies have reported significantly higher concentrations of serum DNA in patients with various types of cancers by a radioimmunoassay method, and have suggested the use of serum DNA in cancer patients as a prognostic tool to monitor the effect of cancer therapy.[37,38] By using a simple colorimetric assay in a representative series of lung cancer patients and controls, we have demonstrated that a quantitative plasma DNA test is a valuable diagnostic tool to discriminate patients from healthy individuals and to detect early recurrence during follow-up. A recent study performed in a group of miscellaneous tumors confirmed these results by using a fluorometric assay and supported a digital single nucleotide polymorphism analysis of allelic imbalances as a sensitive and specific tool for ovarian cancer screening.[26]

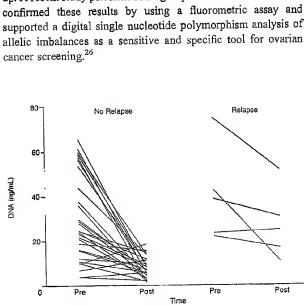

Fig 4. Reduction in DNA level at patient follow-up according to relapse status.

We report here the results of a large case-control study, the first to our knowledge, for validation of free circulating DNA in plasma as a potential lung cancer diagnostic marker. Our results show that real-time quantitative PCR assay, using the *hTERT* gene as a target sequence for quantification of circulating DNA in plasma, has high sensitivity and specificity, as estimated by AUC ROC curves, by analyzing values either as continuous distribution or as selected cutpoints. Furthermore, median concentration in patients (24.3 ng/mL) was almost 8 × the concentration detected in controls (3.1 ng/mL). Although the highest sum of sensitivity (90%), specificity (86%), positive predictive value (90%), and negative predictive value (90%) was obtained with a DNA concentration value of 9 ng/mL (not shown), the CIs around these diagnostic indicators overlap with those of adjacent concentrations. The selection of the optimal cutpoint will therefore have to acknowledge this variability. The value of 25 ng/mL is the only cutpoint shown with sensitivity that does not overlap with that of other cutpoints, although it shows the lowest sensitivity (46%; 95% CI, 36% to 56%). The magnitude of reported ORs proves the strong association between plasma DNA concentration and NSCLC risk, despite wide confidence limits. To our knowledge, similar OR values never were reported previously for any biologic marker and could be of substantial benefit in clinical practice.

We found increased amounts of circulating plasma DNA in samples from any stage and tumor size. This is particularly relevant for small lesions, the systematic detection of which could help reduce lung cancer morbidity and mortality.

One important aspect of our quantitative analysis was the ability to follow longitudinal changes after cancer resection. The data available on 35 cancer patients showed a rapid decrease of circulating DNA values after lung resection. Conversely, no decreasing or increasing levels of plasma DNA identified individuals with recurrence of their disease (24.7 *v* 7.1 ng/mL in cancer-free patients; $P = .002$), suggesting that quantification of plasma DNA might represent a novel approach to monitor surgical patients or assess treatment efficacy after chemoradiotherapy.

To explore the possible modulation of free DNA release by smoking exposure, we analyzed 20 never smokers older than 55 years of age: their median DNA value was 0.61 ng/mL, indicating low amounts of free circulating DNA in unexposed groups.

The origin and mechanism of circulating DNA are not fully understood. In addition to cell lysis, apoptosis, necrosis, and active DNA release have been advanced as possible sources of circulating DNA.[39] To explore the potential mechanisms of tumor DNA release into the bloodstream, we evaluated necrosis, angiogenesis, and proliferation features in all primary tumor samples. Plasma DNA levels in patients were not associated with necrosis, lymphoid infiltration, or growth patterns; or Ki67 or EGFR expression. These data suggest that the mechanism of release of tumor DNA into the bloodstream is not related to the necrotic rate or tumor cell proliferation. Of interest, a significant association with microvessel density suggests a link with tumor angiogenetic status. Because angiogenesis appears to be an early event in lung carcinogenesis,[40,41] plasma DNA quantitative assay could be effective in identifying early but nevertheless angiogenic lung cancers.

In summary, these results highlight a potential value of this DNA-based plasma test for early detection of lung cancer in high-risk individuals, and particularly former heavy smokers. A large study is currently under way with more than 1,000 smoking volunteers to determine whether quantitative detection of plasma DNA might increase the accuracy of spiral CT scan for early detection of lung cancer. Moreover, levels of plasma DNA could help identify high-risk individuals for chemoprevention trials, and could be tested as a potential intermediate biomarker of the efficacy of intervention.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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Detection and quantification of mutations in the plasma of patients with colorectal tumors

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Contributed by Bert Vogelstein, September 16, 2005

The early detection of cancers through analysis of circulating DNA could have a substantial impact on morbidity and mortality. To achieve this goal, it is essential to determine the number of mutant molecules present in the circulation of cancer patients and to develop methods that are sufficiently sensitive to detect these mutations. Using a modified version of a recently developed assay for this purpose, we found that patients with advanced colorectal cancers consistently contained mutant adenomatous polyposis coli (*APC*) DNA molecules in their plasma. The median number of *APC* DNA fragments in such patients was 47,800 per ml of plasma, of which 8% were mutant. Mutant *APC* molecules were also detected in >60% of patients with early, presumably curable colorectal cancers, at levels ranging from 0.01% to 1.7% of the total *APC* molecules. These results have implications for the mechanisms through which tumor DNA is released into the circulation and for diagnostic tests based on this phenomenon.

colorectal cancer | plasma DNA | tumor suppressor gene | circulating DNA | diagnosis

The probability of curing cancers through surgery alone is high in individuals whose primary tumors are detected at a relatively early stage. Such early detection is therefore one of the most promising approaches for limiting cancer morbidity and mortality in the future (1). At present, Pap smears can be used to detect cervical cancers, mammography can detect breast cancers, serum PSA (prostate-specific antigen) levels can signify the presence of prostate cancer, and colonoscopy and fecal occult blood tests can detect colon cancers (2). However, problems with sensitivity, specificity, cost, or compliance have complicated widespread implementation of many of these tests (3–5). Moreover, methods for the early detection of most other cancer types are not yet available.

The discovery of the genetic bases of neoplasia has led to new approaches to detect tumors noninvasively (6–8). Several of these approaches rely on the *ex vivo* detection of mutant forms of the oncogenes and tumor suppressor genes that are responsible for the initiation and progression of tumors. This approach was first used to detect bladder and colon tumors through examination of urine and stool, respectively (9, 10), and has since been used to detect several other tumor types (11–14). Because the mutant genes are not only "markers" for cancer but also the proximate causes of tumor growth (1), they have major conceptual advantages over conventional markers such as fecal occult blood or serum PSA. In particular, conventional markers are not pathogenically involved in the tumorigenic process and are much less specific for neoplasia than are mutations.

The evaluation of patient blood samples for mutant DNA molecules is a particularly attractive approach because such tests could detect many different forms of cancers. Additionally, blood can be easily obtained from patients during routine outpatient visits, and methods for preparing and storing plasma and serum are well known and reliable. Accordingly, numerous studies have attempted to identify abnormal forms or quantities of DNA in plasma or serum (6, 11–15). Unfortunately, the results of many of these studies are contradictory. Some report high detection rates of cancers, and others report very low detection rates, despite the use of similar techniques and patient cohorts. Moreover, several studies have shown that loss of heterozygosity is routinely detectable in circulating DNA, even in patients with relatively nonaggressive tumors. To detect loss of heterozygosity in such samples, the neoplastic cells within a tumor must contribute >50% of the total circulating DNA.

The above studies, although promising, lead to several questions that must be answered to engender confidence in the use of circulating, abnormal DNA as a biomarker of malignancy. First, how many copies of a given gene fragment are present in the circulation in cancer patients? Second, what is the nature of this DNA (e.g., intact vs. degraded)? Third, what fraction of these gene fragments have an abnormal (e.g., mutant) DNA sequence? And, fourth, how does this fraction vary with stage of disease? To answer these questions, it was necessary to develop technologies that could simultaneously quantify the number of normal and mutant DNA molecules in a given sample, even when the fraction of mutant molecules was very small. In the current study, we employ such a technology to investigate circulating DNA in patients with colorectal tumors.

Materials and Methods

Sample Collection, DNA Extraction, and Sequencing. Detailed methods for these procedures are provided in the supporting information, which is published on the PNAS web site.

Real-Time PCR. Primers were designed to generate ≈100-bp amplicons that included one or more mutation sites. A universal tag (5′-TCCGCGAAATTAATACGAC-3′) was added to the 5′ end of either the forward or reverse primer used to generate each amplicon. The sequences of these primers are listed in the supporting information. PCR was performed in 50-μl reactions containing 10 μl of 5× Phusion HF buffer, a 0.2 mM concentration of each dNTP, a 1 μM concentration of each primer, 1:50,000 dilution of SYBR green I (Invitrogen), 1.5 units of Phusion DNA polymerase (NEB, Beverly, MA), and 15 μl of purified plasma DNA (equivalent to 100 μl of plasma) or genomic DNA purified from normal mononuclear cells of the blood of healthy volunteers. The amplifications were carried out with an iCycler (Bio-Rad) under the following conditions: 98°C for 1 min; 98°C for 10 s, 70°C for 10 s, and 72°C for 10 s 3 times; 98°C for 10 s, 67°C for 10 s, and 72°C for 10 s 3 times; 98°C for 10 s, 64°C for 10 s, and 72°C for 10 s 3 times;

Conflict of interest statement: Under a licensing agreement between EXACT Sciences and The Johns Hopkins University, K.W.K. and B.V. are entitled to a share of royalties received by the university on sales of products related to digital PCR. Under a licensing agreement between Agencourt Biosciences Corporation and The Johns Hopkins University, D.D., K.W.K., and B.V. are entitled to a share of royalties received by the university on sales of products related to the use of BEAMing for preparing templates for DNA sequencing. The terms of these arrangements are being managed by The Johns Hopkins University in accordance with its conflict of interest policies.

Abbreviations: APC, adenomatous polyposis coli; PE, phycoerythrin.

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and 98°C for 10 s, 61°C for 10 s, and 72°C for 10 s 30 times. Each reaction was performed in duplicate, and a calibration curve was generated in each 96-well plate by using various amounts of normal human genomic DNA. The concentration of PCR products was determined by using a PicoGreen dsDNA quantification assay (Invitrogen).

BEAMing. A common oligonucleotide (5′-TCCCGCGAAATTA-ATACGAC-3′) was synthesized with a dual biotin group at the 5′ end and with a six-carbon linker (C6) between the biotin and the other nucleotides (Integrated DNA Technologies, Coralville, IA). This oligonucleotide was coupled to streptavidin-coated magnetic beads (MyOne, Dynal, Oslo) according to the protocol described in ref. 16. The water-in-oil emulsions were prepared by modifications of the methods described by Ghadessy and Holliger (17) and Bernath *et al.* (18). For each emulsion PCR, a 240-μl aliquot of an aqueous PCR mix was added to 960 μl of 7% (wt/vol) Abil EM90 (Degussa Goldschmidt Chemical, Hopewell, VA) in mineral oil (Sigma). The aqueous phase contained 67 mM Tris·HCl (pH 8.8), 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 10 mM 2-mercaptoethanol, a 0.2 mM concentration of each dNTP, 0.05 μM forward primer (5′-TCCCGCGAAATTAATACGAC-3′), 8 μM reverse primer, 0.2 units/μl Platinum Taq polymerase (Invitrogen), 3×10^6 per μl oligonucleotide-coupled beads, and 0.1 pg/μl template DNA. The reverse primers are listed in the supporting information. The water–oil mix was vortexed for 10 s and then emulsified for 50 s by using an Ultra-Turrax homogenizer (T25 basic, IKA, Wilmington, NC) with a disposable OmniTip (Omni International, Waterbury, CT) at the minimum speed. The emulsions were aliquoted into 8 wells of a 96-well PCR plate and cycled under the following conditions: 94°C for 2 min; 94°C for 10 s, 58°C for 15 s, and 70°C for 15 s 50 times. After PCR, the emulsions were pooled into a 15-ml tube and demulsified through the addition of 10 ml of NX buffer (100 mM NaCl/1% Triton X-100/10 mM Tris·HCl, pH 7.5/1 mM EDTA/1% SDS). After vortexing for 10 s, the beads were pelleted by centrifugation for 5 min at 4,100 × *g*. The top phase was removed, and the beads were resuspended in 800 μl of NX buffer and transferred to a 1.5-ml tube. The beads were collected by using a magnet (MPC-S, Dynal) and washed with 800 μl of wash buffer (20 mM Tris·HCl, pH 8.4/50 mM KCl). The double-stranded DNA on the beads was converted to single-stranded DNA by incubation in 800 μl of 0.1 M NaOH for 2 min at room temperature. The beads were washed twice with 800 μl of wash buffer, using the magnet, and finally resuspended in 200 μl of wash buffer. Single base extension and flow cytometry were performed as described in the supporting information.

Results

Circulating Mutant DNA Is Degraded. We used real-time PCR or digital PCR to determine the number of total circulating *APC* (adenomatous polyposis coli) genes in 33 patients with colorectal tumors and 10 age-matched donors without any tumors. The number of *APC* gene copies was significantly higher in advanced stage patients (Dukes' D) than in patients with early stage cancers ($P < 0.0001$, Student's *t* test), consistent with previous studies (19, 20). In advanced stage patients, the median number of *APC* gene fragments per ml of plasma was 47,800, whereas the median number was 3,500 and 4,000 for patients with Dukes' A and Dukes' B cancers, respectively (Table 1). There was no significant difference between the number of circulating copies in early stage cancer patients (Duke's A or B), patients with adenomas (4,300 *APC* fragments per ml of plasma), and normal individuals (3,460 *APC* fragments per ml of plasma; range of 1,150–8,280 fragments per ml).

To determine the size of mutant gene fragments in circulating DNA, we analyzed plasma DNA from three patients with advanced colorectal cancers (Dukes' D, metastatic to liver) who were shown to contain *APC* gene mutations in their tumors. By varying the size of the amplicons, it was possible to determine the number of normal and mutant gene fragments by sequencing the PCR products derived from one or a few template molecules (detailed in the supporting information). The size of the amplicons varied from 100 to 1,296 bp and encompassed the mutation present in each patient. The number of total *APC* fragments (WT plus mutant) increased by 5- to 20-fold as the size of the amplicons decreased from 1,296 to 100 bp (Fig. 1*A*). The fraction of mutant molecules was strikingly dependent on size of the amplicon, increasing by >100-fold over the size range tested (Fig. 1*B*).

We conclude that the mutant DNA fragments present in the circulation of cancer patients are degraded compared with the circulating DNA derived from nonneoplastic cells. This conclusion is consistent with previous studies of other tumor types (21, 22) and has important implications for the detection of such mutant molecules.

Development of a Quantitative Assay for Detection of Rare Mutations. The results described above were obtained by sequencing hundreds of PCR products, each derived from one or a few DNA template molecules. In preliminary studies, we found that such digital PCR-based techniques were sufficiently sensitive to detect circulating mutant DNA molecules in patients with advanced cancers but not in patients with early stage cancers. To increase the sensitivity and reliability of these assays, we developed an extension of BEAMing (which derives its name from its principal components: beads, emulsion, amplification, and magnetics) that allowed us to examine many more template molecules in a convenient fashion. The approach consists of four steps. (*i*) Real-time PCR was used to determine the number of total *APC* gene fragments in the plasma sample (Fig. 2*A*, step 1). (*ii*) BEAMing was used to convert the amplified plasma DNA into a population of beads (Fig. 2*A*, steps 2–4). (*iii*) The mutational status of the extended beads was determined by single base extension (Fig. 2*B*). (*iv*) Flow cytometry was used to simultaneously measure the FITC, Cy5, and phycoerythrin (PE) signals of individual beads.

Fig. 3 shows a representative flow cytometry result wherein the interpretation of the profiles was confirmed experimentally. In the example shown, 342,573 beads were analyzed by flow cytometry. The single bead population (295,645) was used for the fluorescence analysis (Fig. 3*A*). Of these, 30,236 exhibited a PE signal (Fig. 3*B*), indicating that they had been extended during the emulsion PCR. The FITC and Cy5 signals reflected the number of beads containing mutant or WT sequences, respectively. Beads containing the WT DNA sequences (30,186) had high Cy5 but background FITC signal ("red beads" in Fig. 3*C*). Beads extended only with mutant DNA sequences (22) had high FITC signals but background Cy5 signals ("green beads"). Twenty-eight had both FITC and Cy5 signals ("blue beads"). Such dual-labeled beads resulted from either the presence of both a WT and mutant template in the droplet containing the bead or an error in the early cycles of the emulsion PCR (see below). These dual-labeled beads were eliminated from analysis, and only homogeneously labeled beads were considered for the enumeration of mutations. Note that this conservative analysis strategy results in a slight underestimation of the fraction of mutations, because it excludes mutants that were present in droplets that also contained one or more WT fragments. Beads in each of these three populations were collected by flow sorting, and single beads from the sort were used as templates in conventional DNA sequencing. All 131 beads subjected to sequencing analysis showed the expected patterns, with examples illustrated in Fig. 3*C*.

Limits to the Sensitivity of Assays for Plasma DNA Mutations. The results described above show that the BEAMing approach can, in principle, detect a very small fraction of fragments containing mutant sequences within a much larger pool of fragments containing WT sequence. Because >50 million beads are used in a single emulsion PCR and flow cytometry can be performed

Table 1. Quantification of *APC* mutations in plasma

Patient no.	Sex/ age, yr	Site	Dukes' stage (tumor node metastasis stage)	Diameter of lesion, cm	Mutation identified in primary tumor (codon)	Fragments per ml of plasma	No. of fragments analyzed	Percentage of mutant fragments, %
1	M/50	Ascending colon	Adenoma	3.0	C4348T (1450)	2,600	2,350	0.002
2	M/67	Descending colon	Adenoma	2.5	C4285T (1429)	5,080	5,080	0.001
3	M/54	Rectum	Adenoma	4.0	G3856T (1286)	4,150	4,150	0.002
4	F/82	Rectum	Adenoma	3.0	4147–4148insA (1383)	1,350	1,350	0.001
5	F/65	Rectum	Adenoma	1.0	C4067G (1356)	4,260	4,260	0.001
6	F/71	Ascending colon*	Adenoma	4.0	G3856T (1286)	4,150	4,150	0.001
7	M/68	Cecum	Adenoma	6.5	C4285T (1429)	4,760	4,760	0.003
8	M/93	Ascending colon	Adenoma	0.8	A4345T (1449)	4,320	4,320	0.001
9	F/78	Ascending colon	Adenoma	3.0	C4216T (1406)	28,570	28,570	0.001
10	F/59	Sigmoid colon	Adenoma	5.0	4661–4662insA (1554)	2,160	2,160	0.002
11	F/73	Ascending colon	Adenoma	5.0	C4348T (1450)	8,000	8,000	0.02
Median/mean						4,300/6,300		0.02*
Mutant plasma samples per samples analyzed								1/11 (9)
12	F/81	Sigmoid colon	A (T2N0M0)	4.0	G4189T (1397)	7,900	12,000	0.01
13	F/75	Sigmoid colon	A (T2N0M0)	2.5	3927–3931del AAAGA (1309)	2,160	2,160	0.001
14	M/60	Sigmoid colon	A (T2N0M0)	3.0	3927–3931del AAAGA (1309)	4,600	6,900	0.04
15	M/79	Right colic flexure	A (T2N0M0)	3.0	4470delT (1490)	4,600	3,696	0.03
16	M/70	Ileocecal	A (T2N0M0)	2.5	4481delA (1494)	6,200	3,105	0.07
17	F/68	Ascending colon	A (T2N0M0)	3.5	C4348T (1450)	2,170	2,170	0.001
18	F/66	Sigmoid colon	A (T1N0M0)	2.5	3927–3931del AAAGA (1309)	1,920	1,920	0.001
19	M/68	Rectum	A (T2N0M0)	5.5	C3907T (1303)	2,300	1,170	0.12
Median/mean						3,500/4,000		0.04/0.04*
Mutant plasma samples per samples analyzed								5/8 (63)
20	F/65	Cecum	B (T3N0M0)	3.5	G4396T (1466)	5,300	5,300	0.002
21	M/71	Sigmoid colon	B (T3N0M0)	3.0	C4348T (1450)	2,100	1,863	0.19
22	M/37	Descending colon	B (T4N0M0)	10.0	C4330T (1444)	5,400	4,887	1.28
23	M/64	Sigmoid colon	B (T3N0M0)	6.5	C4099T (1367)	3,810	3,810	0.001
24	M/72	Sigmoid colon	B (T3N0M0)	3.0	C4012T (1338)	4,800	4,800	0.03
25	F/82	Hepatic flexure	B (T3N0M0)	4.0	C4099T (1367)	3,840	3,840	1.46
26	M/83	Ascending colon	B (T3N0M0)	6.0	4470delT (1490)	1,600	1,404	1.75
27	M/61	Sigmoid colon	B (T3N0M0)	4.0	4260–4261delCA (1420)	4,200	4,200	0.001
Median/mean						4,000/3,900		1.28/0.94*
Mutant plasma samples per samples analyzed								5/8 (63)
28	F/83	Ascending colon	D (T3N2M1)	5.0	4661–4662insA (1554)	230,000	24,857	5.6
29	M/55	Sigmoid colon	D (T3N0M1)	3.0	G3925T (1309)	69,600	1,636	27.4
30	F/33	Descending colon	D (T4N1M1)	5.0	C4067A (1356)	18,000	491	10.5
31	M/64	Sigmoid colon	D (T4N2M1)	6.0	T4161A (1387)	26,000	975	1.9
32	M/56	Rectum	D (T3N2M1)	3.0	4468–4469delCA (1490)	103,200	1,187	18.9
33	F/60	Rectum	D (T3N2M1)	4.0	4059–4060insT (1354)	8,400	850	2.0
Median/mean						47,800/75,900		8.05/11.05*
Mutant plasma samples per samples analyzed								6/6 (100)

*Calculated only for samples in which the percentage of mutant fragments was significantly higher than in control samples (i.e., >0.003%; printed in boldface). M, male; F, female.

at speeds of >50,000 beads per s, the capacity to enumerate such mutations is not limited by the beads themselves. Instead, two other features limit the sensitivity. First, there is a finite number of DNA fragments present in clinical samples. As noted above, this number ranged from 1,350 to 230,000 fragments per ml in the patients with tumors (Table 1) and from 1,150 to 8,280 fragments per ml in control patients, which gives an upper bound to the sensitivity of the assays. For example, a calculation using the Poisson distribution shows that if 4,000 fragments were analyzed, the mutation fraction in circulating DNA would have to be >1 in 1,333 fragments (i.e., 3 divided by the number of total fragments analyzed) for the assay to achieve 95% sensitivity. A second limiting feature is the error rates of the polymerases used for PCR. In our approach, two PCR steps are used: The first is a conventional PCR that employs plasma DNA fragments as templates, and the second is an oil-in-water emulsion PCR that uses the initial PCR products as templates. In the emulsion PCR, errors occurring during the early rounds of PCR can result in

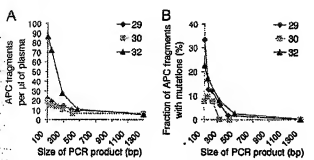

Fig. 1. Effect of the PCR amplicon size on plasma DNA concentration and mutation frequency. (A) The concentration of total *APC* fragments (WT plus mutant) of various sizes was determined by using digital PCR of plasma DNA from three different patients (patients 29, 30, and 32). (B) The fraction of mutant *APC* fragments was determined by digital sequencing of PCR products.

heterogeneous beads containing both WT and mutant sequences. These beads are easily eliminated from consideration, as described in Fig. 3C. However, the errors introduced in the first PCR cannot be eliminated, because they give rise to beads with homogeneous mutant sequences, indistinguishable from those resulting from genuine mutations in the original plasma DNA templates.

The fraction of mutant molecules present after the first PCR equals the product of the mutation rate of the polymerase and the number of cycles carried out. BEAMing provides a quantitative way to determine the error rate of any polymerase used in PCR without requiring cloning in bacterial vectors (M.L., F.D., S.N.G., K.W.K., and B.V., unpublished data). Of 19 different base changes evaluated in normal DNA, the error rates with the polymerase used in the current study averaged 3.0×10^{-7} mutations per bp per PCR cycle and ranged from 1.7×10^{-7} to 6.5×10^{-7} mutations per bp per PCR cycle, depending on the mutation site assessed. As a result, we only scored plasma samples as positive for mutations if their frequency in the sample was significantly higher than the maximum error rate of polymerase found experimentally (i.e., 1.95×10^{-5} after 30 cycles). As a result of the relatively low error rate with the polymerase used, it was the number of molecules present in the original plasma sample, rather than the polymerase error rate *per se*, that limited sensitivity.

These issues suggest that the sensitivity of assays for circulating mutant DNA could be increased in the future by (*i*) the development of new or modified polymerases with reduced error rates and (*ii*) the use of more plasma per assay (i.e., more template molecules).

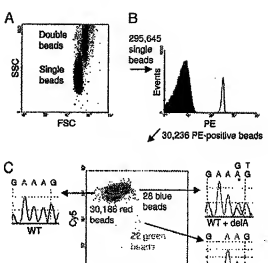

Fig. 3. Processing of flow cytometry data obtained by BEAMing. (A) Dot plot of forward-scatter (FSC) and side-scatter (SSC) signals of beads. (B) Histogram of single beads with regard to PE signal. (C) Dot plot showing the Cy5 and FITC fluorescence intensity profiles of PE-positive beads. The beads clustered in three distinct populations colored red, green, and blue. Sequencing of individual beads sorted from each population showed that the red and green beads contained homogeneous WT and mutant sequences, respectively; the blue beads contained a mixture of WT and mutant sequences.

Quantification of Mutant *APC* Fragments in Plasma from Patients with Colorectal Tumors. Based on the principles derived from the experiments described above, we determined whether fragments of tumor DNA could be detected in patients with colorectal tumors of various types. We selected *APC* gene mutations for this assessment, because >85% of colorectal tumors contain mutations of this gene, irrespective of tumor stage (23). Mutations within codon 1209–1581 of *APC*, containing most previously identified mutations, were evaluated by sequencing of DNA purified from the tumors of 56 patients. Mutations were observed in 33 of these patients (59%), and, as expected, the proportion of tumors with these mutations did not differ significantly among tumors of various stages (see the supporting information).

A BEAMing assay was then designed for each of the mutations identified in the 33 tumors and applied to the DNA purified from the plasma of the corresponding patients (Table 1). In each case, DNA from normal lymphocytes or plasma from patients without

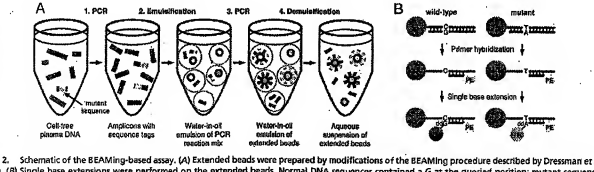

Fig. 2. Schematic of the BEAMing-based assay. (A) Extended beads were prepared by modifications of the BEAMing procedure described by Dressman *et al.* (16). (B) Single base extensions were performed on the extended beads. Normal DNA sequences contained a G at the queried position; mutant sequences contained an A.

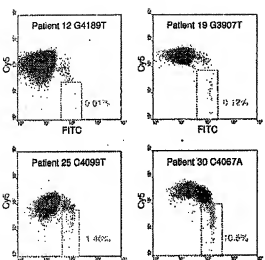

Fig. 4. Examples of flow cytometric profiles of beads generated from plasma DNA (patient 16). CyS and FITC fluorescence intensity profiles of PE-positive beads from four patients are shown. The patients, mutations, and fraction of mutant *APC* fragments are indicated.

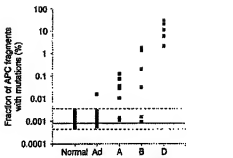

Fig. 5. Fraction of mutant *APC* gene fragments in the plasma of patients with various colorectal tumors (adenomas (Ad) and Dukes' stage A, B, and D carcinomas). In each mutation analyzed, DNA from normal lymphoid cells or plasma DNA from healthy donors was used as a control (Normal). The "mutants" observed in assays with normal cellular DNA represent errors generated during the PCR process rather than mutations present in the template DNA (see text). The red lines represent the mean, minimum, and maximum values of the normal controls.

cancer was used as a negative control. DNA from the tumors of the 33 patients was used as a positive control. All six patients with advanced lesions (Dukes' D, defined as having at least one distant metastatic lesion) were found to contain mutant DNA fragments in their plasma. Among 16 patients harboring cancers with a favorable prognosis (Dukes' A or B, defined as having no lymph node involvement and no distant metastases), 10 (63%) were found to contain mutant DNA fragments in their plasma. In contrast, among 11 patients with large, benign tumors (adenomas), only 1 patient's plasma was found to contain mutant DNA fragments. Representative flow cytometric results are shown in Fig. 4 and summarized in Table 1.

The fraction of mutant molecules found in the plasma of the 17 cases with detectable mutations also varied according to tumor stage ($P < 0.0001$, Fisher exact test). In the advanced cases (Dukes' D), an average of 11.1% (range of 1.9–27%) of the total *APC* gene fragments were mutant. In patients without metastases (Dukes' B), an average of 0.9% (range of 0.03–1.75%) of the plasma *APC* gene fragments were mutant. In patients with lower stage tumors (Dukes' A), the fraction was even lower, averaging 0.04% (range of 0.01–0.12%). And in the one patient with a benign tumor, only 0.02% of the plasma DNA fragments were mutant. The median fraction of positive beads found in the control DNA samples from patients without cancer was 0.0009% (range of 0.003–0.0005%). The mutations in the control samples likely resulted from PCR errors, as noted above.

Table 1 also lists the concentration of total *APC* fragments (WT plus mutant) in these patients' plasma. There was no direct relationship between the concentration of total *APC* fragments and the mutational load. Although patients with advanced cancers tended to have higher concentrations of total *APC* fragments than the other patients, this increase was not due to DNA from neoplastic cells. Furthermore, no correlation was found between tumor burden (volume of primary tumor plus metastatic sites) and either the concentration of *APC* fragments or percentage of mutant *APC* fragments in the circulation.

Discussion

The data described above conclusively demonstrate that *APC* gene fragments from the neoplastic cells of colorectal tumors can be found in the circulation and that the number of such fragments depends on tumor stage. These results have implications for both colorectal tumor biology and for practical diagnostic tests, as discussed below.

Source of Plasma DNA. Previous studies have shown that the total DNA concentration in the plasma of cancer patients is often elevated (19, 20). Our results support this conclusion only in advanced stage patients, in that more total *APC* gene fragments (WT plus mutant) were present in the plasma of patients with Dukes' D cancers than in those with earlier stage tumors. Our results additionally show that this "extra" DNA in advanced stage patients is not derived from the neoplastic cells themselves, because only a minor fraction of the circulating *APC* fragments are mutant, whereas all of the neoplastic cells' *APC* fragments are mutant.

But there are still a large number of mutant DNA fragments circulating in cancer patients. Assuming that the volume of distribution of DNA at steady state is similar to that of oligonucleotides in primates (60–70 ml/kg), an 8% fraction of mutant molecules among 47,800 fragments per ml of plasma (as in Dukes' D patients) would correspond to 1.6×10^7 mutant fragments present in a 70-kg person at any given time (24). The half-life of this tumor DNA is estimated at 16 min, based on the data obtained from clearance of fetal DNA in maternal plasma (25), which translates to $\approx 6 \times 10^8$ mutant fragments released from the tumor each day. For patients with a tumor load 100 g in size ($\approx 3 \times 10^{10}$ neoplastic cells), we thereby estimate that 3.3% of the tumor DNA is fed into the circulation on a daily basis. For a Dukes' B cancer of 30 g in which 1.3% of the 4,000 circulating *APC* fragments per ml of plasma are mutant, the corresponding estimate is that 0.15% of the tumor DNA is fed into the circulation each day.

So how do mutant *APC* gene fragments get into the plasma? Several clues are provided by our data. The ability to get into the circulation was clearly not related to tumor size, because the benign tumors we studied were as large as the cancers (Table 1), yet the former rarely gave rise to detectable mutant DNA fragments. Similarly, there was no significant correlation between the tumor load (including metastatic deposits) and the amount of mutant DNA in the circulation. In contrast, the degree of invasion was indeed correlated with the number of circulating DNA fragments. Those lesions that weren't invasive (benign tumors) did not commonly feed mutant DNA molecules into the plasma. As tumors

invaded through more layers of the intestinal wall in Dukes' B vs. Dukes' A tumors, and through the intestine to distant sites in Dukes' D vs. Dukes' B tumors, the number of circulating mutant DNA molecules progressively increased (Fig. 5).

Another clue is provided by the size of the mutant DNA molecules. The data in Fig. 1 show that mutant sequences are enriched in small DNA fragments and could not be identified at all in fragments of 1,296 bp.

Based on these observations, we propose that the mutant DNA fragments found in the circulation are derived from necrotic neoplastic cells that had been engulfed by macrophages. As tumors enlarge and invade, they are more likely to outgrow their blood supply. Thus, invasive tumors generally contain large regions of necrosis, whereas benign tumors rarely do (26–29). Necrotic cells are not thought to release DNA into the extracellular milieu (30). However, cells that die from necrosis or apoptosis are routinely phagocytosed by macrophages or other scavenger cells. Interestingly, it has been shown that macrophages that engulf necrotic cells release digested DNA into the medium, whereas macrophages that engulf apoptotic cells do not (30). Moreover, the size of the DNA released from macrophages is small (30). All of these observations are consistent with a model wherein hypoxia induces necrosis of tumors, leading to the phagocytosis of tumor cells and the subsequent release of the digested DNA into the circulation. As tumors become more aggressive, the degree of this necrosis increases and the absolute amount of circulating mutant DNA correspondingly rises. Because necrosis involves the killing of neoplastic cells and surrounding stromal and inflammatory cells within the tumor, the DNA released from necrotic regions is likely to contain WT DNA sequences as well as mutant sequences. This phenomenon may explain the increase in total (nonmutant) circulating DNA observed in the plasma of patients with advanced cancers.

Clinical Implications. The ability to detect and quantify mutant DNA molecules in the circulation has obvious clinical importance, and this line of research has been pursued by several investigators. Our results inform the field in several ways. First, it is unlikely that circulating mutant DNA could be used to detect premalignant tumors, based on the fact that we were unable to detect such DNA even in very large adenomas. Second, it is unlikely that loss of heterozygosity detection or other techniques that require a majority of the circulating DNA to be derived from neoplastic cells will allow such detection, at least in colorectal cancers, because the proportion of mutant DNA fragments in plasma was small, averaging only 11% of the total DNA fragments even in large, metastatic cancers.

On the positive side, our data show that even relatively early cancers give rise to circulating mutant DNA fragments that can be detected with sufficiently sensitive and specific assays. In fact, >90% of cancers that had not yet metastasized gave rise to detectable mutant fragments in plasma. Even Dukes' A tumors, which are by definition barely invasive, were detectable with BEAMing-based assays. Virtually all Dukes' A tumors and most Dukes' B tumors can be cured with conventional surgery alone, without the need for adjuvant therapies (31).

In practical terms, plasma-based assays for mutant DNA fragments are inferior in several ways to more conventional techniques for early colorectal cancer detection. Colonoscopy is the gold standard, with sensitivity rates of >80% for adenomas and >90% for cancers (32). In particular, adenomas detected by colonoscopy can often be removed through the colonoscope, alleviating the need for surgery. Unfortunately, a variety of issues limits the widespread applicability of colonoscopy (either conventional or virtual) to the screening of asymptomatic patients (3, 5, 33), a fact that has stimulated the development of noninvasive technologies. One of the most promising of these noninvasive technologies is the analysis of fecal DNA for mutations (34). Because of the frequent presence of mutant DNA molecules in feces from both adenomas and early cancers, fecal DNA analysis is superior to plasma with regard to sensitivity. However, plasma-based assays have potential advantages with regard to ease of implementation and compliance.

For many tumor types, there are currently no alternative methods for presymptomatic diagnosis, unlike the case with colorectal cancers. In these other tumor types, the evaluation of circulating DNA could be particularly useful. Even if such assays could detect only a fraction of patients with treatable cancers, much morbidity and mortality could be averted.

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DETECTING LUNG CANCER IN PLASMA WITH THE USE OF MULTIPLE GENETIC MARKERS

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Recent studies have demonstrated the possibility to detect genetic changes in plasma DNA of cancer patients. The goal of this study was to validate a panel of molecular markers for lung cancer detection in plasma DNA. Three markers, p53, FHIT and microsatellite alterations at loci on chromosome 3, were used to detect mutations in tumor and plasma DNA of 64 stage I–III non small cell lung cancer patients. p53 mutations were studied by direct sequencing of exons 5 through 8 in tumor DNA and by plaque hybridization assay and sequencing in plasma DNA. Allelic losses were evaluated by fluorescent PCR in tumor and plasma DNA. p53 genomic mutations were detected in 26 (40.6%) of 64 tumor DNA samples and the identical mutation was identified in plasma of 19 (73.1%) of them. Microsatellite alterations at FHIT and 3p loci were observed in 40 (62.5%) tumors and in 23 (35.9%) plasma samples. Of the 40 patients showing microsatellite alterations in tumors, 19 (47.5%) displayed the same change in plasma DNA. At least 1 of the 3 genetic markers (p53, FHIT and 3p) was altered in plasma of 51.6% of all patients and 60.7% of stage I patients. Moreover, genetic markers in plasma identified 29 of 45 (64.4%) of all stages and 15 of 22 (68.2%) of stage I patients whose tumors had an alteration. These results provide the proof of principle that plasma DNA alterations are tumor-specific in most cases and support blood testing as a noninvasive strategy for early detection.
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Key words: *lung cancer; early detection; genetic markers; p53; FHIT*

Lung cancer is one of the leading causes of cancer mortality in the world, especially in developed countries.[1] A major problem in lung cancer is the lack of clinically efficient noninvasive methods for early detection and screening of asymptomatic high-risk individuals.

In the past, the most common screening techniques, such as chest radiography and sputum cytology, were unable to reduce the mortality.[2] Recent results achieved by spiral CT have opened new prospects for significant reduction of lung cancer mortality but proper selection of high-risk population and differential diagnosis are critical elements.[3] In fact, even though for stage I lung cancer patients surgical resection can achieve a 60–70% 5-year survival, over 70% of cases are detected in stage II–IV patients where survival is poor.[4] Thus, the development of novel molecular methodologies is needed to facilitate early detection of lung cancer. Lung cancer is associated with a variety of genetic alterations, including p53[5] and K-ras mutations,[6] inactivation of the fragile histidine triad (FHIT) gene,[7,8] allelic imbalances at multiple chromosomal loci[9,10] and aberrant promoter methylation of several genes, mainly $p16^{INK4a}$.[11] Most of these changes have also been described in premalignant lesions and early phases of lung carcinogenesis.[12] The use of sensitive molecular techniques has enabled the detection in the plasma of lung cancer patients of the same genetic alterations observed in their tumors.[13–15] In addition, several studies have demonstrated the presence of significantly higher concentrations of circulating DNA in the plasma/serum in patients with different types of cancer,[16–18] including primary or recurrent lung cancer.[20] Thus, quantification of plasma DNA and characterization of specific molecular changes could represent useful biomarkers of lung cancer. In an attempt to validate a grid of molecular genetic markers detectable in plasma DNA of lung cancer patients, we analyzed a series of 64 patients with stage I–III non small cell lung cancer (NSCLC), focusing our attention on 3 very common alterations: p53, FHIT and allelic imbalances affecting other 4 loci on 3p. The ultimate goal of the study was the validation of molecular approaches that might be useful for an effective early detection and monitoring of NSCLC.

MATERIAL AND METHODS

Pathologic and immunohistochemical methods

Clinical and pathologic data are illustrated in Table I. There were 38 squamous cell carcinomas, 19 adenocarcinomas, 5 large cell carcinomas, and 2 non small cell carcinomas not further defined, Stage I accounted for 45%, stage II for 27% and stage III for 28% of the tumors. For immunohistochemical analysis, formalin-fixed and paraffin-embedded samples obtained at surgery were investigated for p53 and Fhit markers according to previously refined methods.[21] All cases were evaluated blindly without knowledge of the patients' identity, pathologic diagnosis, clinical outcome or plasma DNA status. The percentage of p53 and Fhit-immunoreactive tumor cells was evaluated by scoring a minimum of 1,000 tumor cells in representative fields of immunostaining.

Samples collection and DNA isolation

A consecutive series of 64 NSCLC patients admitted from 1995 to 1997 at the Royal Brompton Hospital, London, was analyzed (Table I). All patients gave informed consent to have their banked specimens analyzed in future molecular studies. All tumor specimens were surgically resected and immediately stored at −140°C. Peripheral blood samples were obtained from the patients on the day of admission and collected in lithium heparin. Plasma was immediately separated from the cellular fraction by centrifuging twice at 900*g* for 10 min at 4°C. The resulting supernatant (plasma) and 2 ml of whole blood were frozen at −80°C. DNA was extracted from tissues, plasma and blood cells samples by using QIAamp DNA Mini Kit (Qiagen, Chatsworth, CA) according to

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TABLE I - PATIENTS FEATURES (n = 64)

	n	%
Age		
Median	64	
> 60	42	66
Female	18	28
Smokers	62	97
Mean ciga/day	24	
> 20 ciga/day	28	44
Type		
Squamous	38	59
Adeno	19	30
Other	7	11
Stage		
I	29	45
II	17	27
III	18	28
Surgery		
Pneumonectomy	18	28
Lobectomy	40	63
Segmentectomy	2	3
Mediastinal biopsy	4	6

the tissue protocol and blood and body fluids protocol; 1,000 µl of plasma were purified by 5 passages on the same column (Qiagen) and the resulting DNA was eluted in 50 µl of sterile bidistilled water and stored at −20°C. Tumor and whole blood DNA concentrations were estimated by spectrophotometry.

p53 mutations detection by polymerase chain reaction and direct sequencing

A 1,672 bp fragment of the TP53 gene, exon 5 through exon 8, was amplified from 64 available primary tumor DNAs by PCR (GeneAmp PCR system 9700, PE, Applied Biosystem, Foster City, CA); 100 ng of DNA purified from primary tumor were used for PCR amplification, performed using the following primers (from MWG oligo) located in the introns flanking exon 5 and exon 8: 312 exo5 (sense), 5'-TTCAACTCTGTCTCCTTCCT-3'; 8.3 exo8 (antisense), 5'-AAGTGAATCTGAGGCATAAC-3'. The resulting 1,672 bp fragment of the TP53 gene was then used as a template to sequence separately the different exons, 5, 6, 7 and 8. PCR amplification was performed as follows: 2.5 µl of 10 × buffer II gold PE, 2.5 µl of 2.5 mM $MgCl_2$, 1 µl of 2.5 mM dNTP mix, 2 µl of each primer (312 and 8.3) 20 µM, 0.3 µl of 5 units/µl Amplitaq Gold (Applied Biosystem) and 37.7 µl of sterile water. The final volume was 50 µl. Samples were then processed through an incubation of 10 min at 95°C and 40 cycles consisting of 40 sec at 94°C, 40 sec at 62°C annealing temperature and 2 min at 72°C, for the first 10 cycles, and 40 sec at 94°C, 40 sec at 60°C, 2 min at 72°C for the last 30 cycles with a final extension at 72°C for 5 min. The PCR products were purified (Qiaquick PCR purification Kit, Qiagen) and exons 5–8 were sequenced individually by cycle sequencing using appropriate primers located in the introns.

Plaque hybridization assay

The search for p53 mutations in plasma was performed in 26 patients (27 mutations) found to have p53 mutation in their primary tumor with oligonucleotide plaque hybridization assay. The exon containing the p53 mutation found in the primary tumor was amplified from plasma DNA by PCR with the appropriate programs and primers for each exon; 5 µl of plasma DNA were used for the amplification in a final volume of 50 µl. The fragment was then purified and cloned in a pGEM-Teasy vector (Promega, Madison, WI). After ligation, carried out at RT for 1 hr, the transformation in bacterial cells was performed overnight at 37°C and between 1,000 and 3,000 clones were transferred to nylon membranes (Stratagene, La Jolla, CA).

The membranes were then hybridized with P^{32} end-labeled oligonucleotide probes specific for the p53 mutation identified in the primary tumor as previously described. As a positive control, the primary tumor was used; as a negative control, a plasma DNA sample without mutation was used. Hybridizing plaques indicated the presence of mutation of p53. The positive plaques were picked up and sequenced to confirm the mutation. The ratio of mutant to total white colonies ranged from 1/50 to 1/700. In the negative control, no positive plaques were found. For some plasma samples, where the mutation was not confirmed by sequence even in the presence of conspicuous hybridized plaques, other techniques such as restriction endonuclease analysis (REA) and mutation allele-specific amplification (MASA) were used.

Restriction endonuclease analysis

REA was performed only in plasma samples that had a selective mutation, arginine-to-serine substitution,[22] identified as a hotspot mutation in lung cancer, located in exon 7 of the p53 gene. The plasma samples were analyzed by REA as follow: 5 µl of purified plasma DNA were used to amplify exon 7 of p53 in a final volume of 50 µl with the use of previously described conditions with specific primers located in the introns. After incubation at 95°C for 10 min, the PCR consisted of different steps: 30 sec at 95°C, 30 sec at 63°C, 1 min at 72°C for 10 cycles, 30 sec at 95°C, 30 sec at 62°C, 1 min at 72°C for 30 cycles, followed by a final extension at 72°C; 10 µl of PCR product were digested with *Hae*III (New England Biolabs, Beverly, MA) and the amplification products were visualized on 3% agarose gel with ethidium bromide. Two major fragments of 92 and 66 bp and several small fragments from wild-type sequence were clearly identified on the gel, because the restriction endonuclease cleaved a GG/CC sequence between codon 249 and 250 of exon 7, whereas the presence of an uncleaved band of 158 bp was indicative of the mutation in codon 249, since the enzyme was not able to cut the GG/CC sequence that is destroyed by the mutation (AGG-to-AGT). The complete cleavage was ensured by the absence of the 254 bp fragment. To enhance the sensitivity of our test, the digested product was subjected to an additional PCR reaction to amplify the mutant PCR product selectively; 5 µl of digested products were used for a second amplification using the following nested primers located in exon 7: Nes1 (sense), 5'-AGGCCCACTGGCCTCCTT-3', and Ne2c (antisense), 5'-TGTGCAGGGTGGCAAGTGGC-3'. The final volume was 50 µl. The samples were processed through 30 cycles consisting, after incubation at 95°C for 10 min, of 30 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C, followed by a final extension at 72°C for 5 min. In this case, a fragment of 182 bp was obtained; 15 µl of PCR product were then digested with restriction endonuclease *Hae*III for 1 hr at 37°C in a final volume of 20 µl. Two fragments, of 92 and 66 bp, representative of wild-type sequence and one undigested enriched 158 bp fragment, indicative of the mutation arginine-to-serine in codon 249 of exon 7, were clearly identified on the gel. In the positive control (a tumor sample carrying the mutation), the 158 bp fragment was clearly detected, whereas the negative control (a sample from plasma DNA of a healthy donor) did not show the 158 bp fragment.

The sensitivity of this test was calculated by serially diluting a control DNA containing the mutation at codon 249 from 1:10 to 1:10,000. The presence of the uncleaved 158 bp fragment was still visible at ratio of 1:10,000. To confirm the presence of the mutation, these fragments were separated on a 3% agarose gel stained with ethidium bromide, transferred to a nylon membrane (Hybond-N$^+$ Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized with P^{32} end-labeled oligonucleotide probes containing the p53 mutation at codon 249. The presence of a hybridized 158 bp band in plasma confirmed the mutation.

Mutation allele-specific amplification

One case carrying a deletion of a conspicuous number of bases (27 bp) was analyzed by MASA. The amplification was performed with primers centered upstream and downstream the deletion. The reaction mixture contained 5 µl of DNA, 5 µl of 10 × buffer, 2.5 mM $MgCl_2$, 1 µl dNTP mix, 2 µl of each primer 20 µM, 0.3 µl of 5 units/ml Amplitaq Gold and 37.7 µl of sterile water. The final

volume was 50 µl and the samples were processed through 30–35 cycles at an appropriate annealing temperature for each primer. As a positive control, the corresponding tumor DNA carrying the deletion was used; as a negative control, DNA from lymphocytes of a healthy donor was used. The PCR products were then visualized on a 3% agarose gel stained with ethidium bromide. The presence of an amplified fragment confirmed the deletion in plasma DNA.

Microsatellite analysis

The analysis of microsatellite instability and loss of heterozygosity was performed by studying microsatellite alterations at loci at 3p14.2 (D3S1300, FHIT locus), 3p21 (D3S1289), 3p23 (D3S1266), 3p24.2 (D3S2338), 3p25-26 (D3S1304) that are hotspots of deletions in lung cancer. The sequences of nucleotide markers for microsatellite analysis are available through the Genome Database.

A total of 30 ng of tumor and lymphocyte DNA were used for the analysis; 2 30 ng of purified DNA was used for PCR amplification of plasma by using primer pairs synthesized with FAM, HEX or NED fluorescent labels (PE Applied Biosystems ABI Prism Linkage Mapping Set). PCR protocol was as follows: Buffer II gold PE (10 ×) 1.5 µl, $MgCl_2$ (2.5 mM) 1.5 µl, dNTP mix (2.5 mM) 0.2 µl, labeled primer mix (10 µM) 1 µl, AmpliTaq Gold (5 U/µl) 0.12 µl, sterile water 9.8 µl. Final volume of the reaction was 15 µl. Samples were processed in a GeneAmp PCR system 9700 thermal cycler through 45 cycles, each cycle consisting of 10 sec at 96°C, 30 sec at 55°C annealing temperature, 3 min at 70°C. Pools of the fluorescent PCR products for each clinical specimen were separated electrophoretically on a 5% polyacrylamide gel and analyzed by laser fluorescence using ABI Prism DNA Sequencer (377 PE Applied Biosystem) equipped with GeneScan TM 2.1 software. Loss of heterozygosity (LOH) and the presence of allele shifts indicating genomic instability are recorded in the various samples and compared with the profiles obtained in DNA from normal peripheral lymphocytes. LOH was scored when a reduction of at least 30% of allele intensity in the experimental sample was seen. All the DNA samples with microsatellite alterations were amplified at least twice to rule out PCR artifacts or sample contamination. In the presence of allelic imbalance in plasma, increasing amounts of plasma DNA were used in the PCR reaction in order to exclude unreliable alleletyping.

Statistical analysis

Qualitative data are presented as frequencies and/or percentages and compared using chi-square test or Fisher's exact test. Exact 95% confidence intervals for proportions were calculated using the binomial distribution. An association was considered statistically significant if the corresponding p-value was ≤ 0.05.

RESULTS

p53 mutations, FHIT and 3p LOH analysis in tumor and plasma samples

Table II records the frequency of molecular changes in tumor and plasma. The frequency and type of p53 genomic mutations in tumor samples detected by exon 5–8 amplification and direct sequencing are reported in Table III. Twenty-six out of 64 (40.6%; 95% confidence interval = 28–54%) samples studied showed the presence of genomic mutations in the coding sequence of the p53 gene. Two different mutations in exons 5 and 6 were found in one tumor sample. Twenty-three mutations were missense and 4 were null (Table III). The analysis of microsatellite alterations at 5 loci on 3p showed allelic imbalances affecting at least 1 locus on 3p in 40 of 64 tumors (62.5%; CI = 49–74%). The frequency of alterations at any single locus was D3S1300 (3p14.2), 22/56 (39.3%); D3S1289 (3p21), 15/54 (27%); D3S1266 (3p23), 20/50 (40%); D3S2338 (3p24.2), 22/54 (44%); D3S1304 (3p25-26), 24/53 (45%).

The search for p53 mutations in plasma was done in 26 patients (27 mutations) displaying p53 mutation in the tumor sample by using direct sequencing analysis, plaque hybridization assay (Fig. 1) and sequencing, REA (Fig. 2) and MASA. In 19 of 26 (73.1%; CI = 52–88%) plasma DNA analyzed, we detected, by using one or more of the assays described above, the same p53 mutation identified in the corresponding tumor.

Plasma samples from 64 patients who were informative (*i.e.*, heterozygous) for at least one locus on 3p were evaluated for the presence of microsatellite changes. Twenty-three of 64 plasma samples (35.9%; CI = 24–49%) showed alterations at one or more 3p loci (Table II). Of the 40 patients having microsatellite changes in the tumors, 19 (47.5%; CI = 31–64%) had a corresponding alteration in plasma DNA. In 4 plasma samples, a microsatellite alteration was observed in the absence of a corresponding tumor alteration. Nevertheless, the presence of FHIT and 3p LOH alterations in plasma samples was significantly associated with FHIT and 3p LOH alterations in tumor specimens ($p = 0.02$ in both cases, using Fisher's exact test).

When plasma samples were grouped according to alterations found in any genetic markers (FHIT, 3p, p53), tumor-specific changes were detected in 33 of 64 (51.6%; CI = 39–64%) of all patients and in 17 of 29 (58.6%; CI = 39–76%) of stage I patients. Moreover, genetic markers in plasma identified 29 of 45 (64.4%; CI = 49–78%) of all stages and 15 of 22 (68.2%; CI = 45–86%) of stage I patients whose tumors had a detectable alteration at any of the 3 markers. The frequency of molecular alterations in p53, FHIT or 3pLOH, whether in tumor or in plasma, was not statistically different when compared by stage, histology or age of the patients (data not shown).

TABLE II - FREQUENCY OF p53 MUTATIONS, FHIT AND 3p LOH IN TUMOR AND PLASMA SAMPLES FROM NSCLC PATIENTS

	Tumor	Plasma	Plasma[a]
p53 mutations	26/64 (40.6%)		19/26 (73.1%)
FHIT LOH[b]	22/56 (39.3%)	9/56 (16.1%)	7/22 (32%)
3p LOH[c]	40/64 (62.5%)	23/64 (35.9%)	19/40 (47.5%)
Any change	45/64 (70.3%)	33/64 (51.6%)	29/45 (64.4%)

[a]Fraction of plasma samples with alterations from patients showing a corresponding change in the tumor.– [b]In specimens unable to determine presence or absence (noninformative).– [c]Number of cases containing at least 1 microsatellite alteration in the panel of 5 markers analyzed.

TABLE III - CHARACTERISTICS OF p53 MUTATIONS

Number of cases with mutation	Exon	Mutation	Codon	Amino acid change
1	5	G→A	135	Cys→Tyr
1	5	Insertion C	151	Frameshift
1	5	G→T	154	Gly→Val
2	5	G→T	157	Val→Phe
2	5	C→T	159	Ala→Val
1	5	Deletion 27 bp	159	Frameshift
1	5	Insertion CC	161	Frameshift
1	5	A→T	168	Cys→Tyr
1	6	Deletion 5 bp	187	Frameshift
1	6	T→A	216	Val→Glu
1	6	T→G	218	Val→Gly
1	6	A→G	220	Tyr→Cys
2	7	T→G	234	Tyr→Cys
1	7	G→T	237	Met→Ile
1	7	C→T	248	Arg→Leu
3	7	G→T	249	Arg→Ser
1	7	A→T	249	Arg→Trp
1	8	G→A	273	Arg→His
1	8	G→A	275	Cys→Tyr
1	8	A→T	280	Arg→Stop
1	8	C→T	282	Arg→Trp
1	8	G→T	294	Glu→Asp

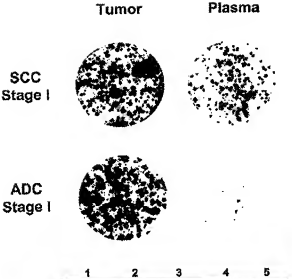

FIGURE 1 – Analysis of p53 mutations by plaque hybridization assay in plasma DNA from lung cancer patients showing variable number of mutant p53 alleles in stage I tumors. Nylon membranes were hybridized with mutant-specific oligonucleotides. Representative hybridizing clones were selected for sequencing. As a negative control, plasma DNA from individuals without p53 mutation was used in each experiment.

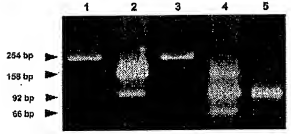

FIGURE 2 – Detection of plasma Ser-249 p53 mutation in an NSCLC patient with REA. Lane 1, amplification of exon 7 from tumor sample by PCR; lane 2, fragment of exon 7 from tumor sample digested with *Hae*III. The 2 fragments of 92 and 66 bp originate from wild-type, because the restriction endonuclease cleaves a GG/CC sequence between codon 249 and 250 of exon 7; the presence of the 158 bp fragment is indicative of the mutation in codon 249, since the enzyme is not able to cut the GG/CC sequence that is destroyed by mutation. Lane 3, amplification of exon 7 from plasma sample by PCR; lane 4, fragment of exon 7 from plasma sample digested with *Hae*III. The 2 fragments of 92 and 66 bp are indicative of wild-type sequence, whereas the 158 bp fragment is indicative of mutation. Lane 5, control subject. The presence of only the 2 fragments of 92 and 66 bp is indicative of wild-type sequence.

TABLE IV – COMPARISON OF IMMUNOHISTOCHEMICAL AND MOLECULAR DETERMINATIONS IN TUMOR

	Immunohistochemical (n = 64)	Molecular (n = 36)	Both[a] (n = 36)
p53	37 (58%)	6 (41%)[b]	43 (67%)[b]
FHIT	47 (73%)	22 (39%)[b]	43 (77%)[b]
Both[a]	55 (86%)	31 (55%)[b]	51 (91%)[b]

[a]At least one test positive. [b]Assay done in 64 specimens.

Immunohistochemical analysis of p53 and fhit proteins

Positive p53 immunostaining (> 10% reactive cells) was detected in 37 of 64 (57.8%; CI = 45–70%) samples. Twenty of 26 (76.9%; CI = 56–91%) cases showing p53 genomic mutation in exons 5–8 displayed positive p53 immunostaining compared to 17 of 38 (44.7%; CI = 29–62%) tumors without p53 mutation (p = 0.02, Fisher's exact test). In 3 samples, in spite of the presence of missense mutations in exon 6 (1 case) and exon 8 (2 cases), lack of p53 immunoreactivity was observed. Overall, only 20 (54%; CI = 37–70%) of the 37 p53-overexpressing tumors that were also analyzed for genomic mutations showed underlying p53 gene mutations in exons 5–8, suggesting alternative mechanism for p53 protein stabilization in tumor samples.

Since in 39% of the tumor samples allelic imbalances at the D3S1300 locus located in intron 5 of the FHIT gene were observed, we analyzed Fhit protein expression by immunohistochemistry in tumor sections. Complete absence of Fhit protein immunostaining was recorded in 47 of 64 (73.4%; CI = 61–84%) tumors. Of the 17 Fhit protein-positive cases, 2 showed reduced immunoreactivity, with only 10% of cells showing weak immunostaining. Twenty-one of 22 (95.5%; CI = 77–99%) tumors showing FHIT LOH also displayed complete absence of Fhit protein expression compared to 21 of 34 (61.8%; CI = 43–78%) tumors without FHIT LOH (p = 0.005, Fisher's exact test).

No statistically significant association between p53 and Fhit protein expression was observed in tumor specimens, whereas tumors showing either FHIT LOH or 3p LOH displayed a significantly higher frequency of p53 genomic mutations. In fact, in samples with 3pLOH, 21 of 40 exhibited p53 mutations (52.5%; CI = 36–68%), whereas in samples without 3p LOH, 5 of 24 showed p53 mutations (20.8%; CI = 7–42%; p = 0.018, Fisher's test). Similarly, in samples with FHIT LOH, 12 of 22 exhibited p53 mutations (54.5%; CI = 32–76%) and only 9 of 34 samples without FHIT LOH showed p53 mutations (26.5%; CI = 13–44%; p = 0.034, chi-square test).

The combined frequency of p53, FHIT and 3p microsatellite alterations in tumors detected by both immunohistochemical and molecular assays was 93.7% (60 of 64 patients; CI = 85–98%), whereas by molecular assays it was only 70.3% (45 of 64 patients). As illustrated in Table IV, immunohistochemical essays revealed a higher prevalence of p53 and of Fhit alterations in tumor compared to mutational analysis, whether considered individually or combined, and in agreement with high values previously reported.[23]

DISCUSSION

Several studies have provided the evidence that tumor-like DNA is present in plasma of cancer patients and can be detected with sensitive techniques.[1,22,24] Our results confirm and extend at other 3p loci the data previously reported[14] regarding the presence of microsatellite changes at FHIT locus in plasma DNA of lung cancer patients. Moreover, we report here for the first time a p53 mutational analysis in plasma samples performed with sensitive molecular assays.

The choice of the 3 genetic targets, p53, FHIT and 3p loci, was motivated by the consensus on these changes as the most frequent

and earliest alterations in lung carcinogenesis, thus fulfilling the criteria for optimal biomarkers. Two other major molecular changes, $p16^{INK4A}$ promoter hypermethylation and K-ras mutations, were already analyzed by our group in a different series of lung tumor and plasma samples.[13] While $p16^{INK4A}$ methylation resulted as a promising marker, being detectable in 54% of plasma samples, K-ras mutations were not identified in any of the plasma sample analyzed, precluding the use of K-ras as a molecular marker in plasma-based molecular alteration detection for NSCLC.

Combining 5 markers located on chromosome 3p (including FHIT), we observed that 62.5% of patients showed allelic imbalances in tumor samples and 35.9% in plasma samples, whereas 47.5% of the cases showing tumor alteration displayed a corresponding change in plasma DNA. The theoretical sensitivity of the microsatellite assay ranges from 1:100 to 1:200.[25]

p53 mutations in tumors, analyzed by PCR and direct sequencing, identified 40.6% of the patients. However, the frequency of p53 mutations in plasma samples using this technique was very low (less than 1%) due to the coexistence of tumor and normal DNA in plasma that hampers the detection of single-base mutations. The lack of p53 mutations in normal DNA indicates that the mutations identified in plasma samples by more sensitive techniques, plaque assay and, in specific cases, REA and MASA are neither germline nor polymorphisms. Moreover, the p53 mutations detected in our cases were already reported in tumors of different types, including lung (http://www.iarc.fr/p53/).[26] Plaque hybridization assay is a technique with high sensitivity (1:1,000–1:10,000) that has been successfully used to identify p53 mutations in cytologic samples.[27] In the latter study, 39% of lung cancer patients analyzed carried in their bronchoalveolar lavage the same mutation identified in the tumor sample, whereas only 14% of the patients showed microsatellite changes. In our study, the sensitivity of p53 mutation detection in plasma with the use of plaque assay, REA and MASA raised from < 1% to 73%.

With the use of sensitive techniques, the efficiency of p53 mutation detection in plasma seems thus higher than microsatellite alterations assay and the combination of the 3 markers (p53, FHIT, 3p) is able to detect alterations in plasma DNA of 51.6% of all patients and 60.7% of stage I patients. Moreover, genetic markers in plasma identified 64.4% of all stages and 68.2% of stage I patients whose tumors had a detectable alteration in 1 or more of these 3 markers supporting a possible use of molecular assays in plasma for detection of early stage lung cancer.

The genetic analyses used in this study showed that alterations in the plasma matched exactly those found in the primary tumors of the corresponding patients in 29 of 45 (64.4%) patients. In 4 patients, microsatellite alterations were detected in plasma only and even though these changes were confirmed in separate experiments, they may nevertheless represent artifactual imbalances due to the low amount of DNA in the PCR reaction. However, these discordant findings could also reflect intratumoral clonal heterogeneity and biased tissue sampling as previously reported.[28] Nevertheless, since 35.5% (16 of 45) of the patients with alterations in tumors did not have a detectable mutation in 1 of the 3 markers in their plasma DNA, there is a clear need to improve sensitivity of using mutations in plasma DNA to test for lung cancer. On the other hand, the proportion of tumors detected with the 3 genetic markers used in this study is 70% and nearly 30% of the tumor specimens analyzed resulted negative for the presence of any type of the molecular changes. To be able to detect a higher prevalence of genetic changes in tumors, alterations in other genes/markers would have to be assessed.

A major limitation of studying p53 gene mutations by plaque assay, REA or MASA resides in the fact that these molecular assays require the previous knowledge of the mutation present in the tumor. The distribution of p53 mutations in our patients included many different types of mutations scattered all along the DNA binding domain of the p53 protein, from exon 5 to exon 8, although a prevalence of mutations affecting exon 5 ($n = 10$) compared to the other exons ($n = 16$) was observed. As already reported in lung cancer from smokers,[5] we found a predominance of G-to-T transversions that were present in 12 of 27 (44%) of our patients. A frequent p53 mutation identified as a hotspot in hepatocellular carcinoma in a population exposed to aflatoxin B is an arginine-to-serine substitution at codon 249 (Ser-249).[29] Ser-249 mutation is nevertheless reported in 200 of the 10,385 p53 mutation described in human cancer (http://www.iarc.fr/p53/),[26] and 25% of these Ser-249 mutations were found in lung cancer patients. Previous studies have also shown that benzo(a)pyrene-diol-epoxide induces guanine adducts at specific hotspots of the p53 gene, codons 157, 248, 249, 273, in normal human bronchial epithelial cells.[30,31] In the present study, we detected low frequency of mutations in codon 157 (2 cases) and a higher frequency in codons 248–249 (5 cases). Overall, only 7 (24%) of the 26 p53 mutations detected in our patients reside in the reported hotspots, suggesting that a thorough mutational screening is needed in order to use p53 as an informative marker in biologic fluids. However, the p53 mutational analysis could be automated by using a high-throughput format in order to reduce time and costs. Since plaque assay is based on the discrimination of p53 mutation on a single molecule basis through oligonucleotide hybridization, new nanotechnology tools such as DNA microarrays could be applicable for screening purposes, allowing the achievement of good sensitivity with this approach.

In conclusion, the sensitivity of molecular assays either in tumors or in plasma still remains the limiting step for a routine use of molecular markers in clinical practice. Nevertheless, the establishment of a panel of tumor-specific molecular markers detecting 100% of lung tumors undoubtedly represents the ultimate target, although for the time being it appears a difficult achievement. Specificity of molecular assays also needs testing in healthy individuals and we have previously reported that, using the same methods described in this study, microsatellite alterations in plasma DNA were not found in a control group of 43 individuals (including 8 smokers), indicating a very low occurrence of false positive results. Furthermore, recently we could also find good sensitivity and specificity of a real-time quantitative PCR assay in plasma DNA in a case-control study of cancer patients and asymptomatic chronic smokers (data not shown). It is likely that the combination of quantitative and qualitative molecular assays on plasma DNA, developed by high-throughput platforms, will improve the noninvasive approach to lung cancer detection.

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EDITORIAL

Early Detection of Lung Cancer Using Serum RNA or DNA Markers: Ready for "Prime Time" or for Validation?

THERE ARE no established methods to screen smokers or other high-risk subjects for lung cancer. Previous studies of sputum cytolgy or annual chest x-rays showed no benefits in lung cancer mortality reduction, despite improved survival for detected cases.[1] Previous studies of serum protein markers, such as carcinoembryonic antigen and other tumor antigens, have failed to yield sufficient sensitivity or specificity for routine screening use.[2] More recent studies have reported the presence of DNA markers in the serum of patients with a variety of cancers, including lung cancers.

In this issue of the *Journal of Clinical Oncology*, Sozzi et al[3] present the results of the largest of such studies. Using real-time quantitative polymerase chain reaction (PCR) and human telomerase catalytic component (hTERT) primers, they report elevated (> 15 ng/mL) levels in 78% of lung cancer patients, compared with zero controls. Those reports are an important step forward, but much remains to be done before such tests become standard. A PubMed search for serum DNA markers and lung cancer revealed the studies summarized in Table 1.[3-16] These studies can be divided into studies measuring: (1) total DNA[3-5]; (2) gene expression levels using quantitative PCR techniques[5-7]; (3) methylation of the promoter of various tumor suppressor genes, alone or in combination[8-11]; (4) microsatellite alterations using several markers, alone or in combination[4,10,12-16]; and (5) mutations in specific oncogenes such as *K-ras*.[9,10] These studies have several common features. First, the case numbers are small, varying from 16 to 100. Second, the number of control cases is even smaller (n = 0 to 100), and the controls were not matched for obvious clinical features such as age, sex, smoking history, and pulmonary function. Third, abnormalities were found in 25% to 78% of the cases, but in only 0% to 12% of the controls. Fourth, standardized methods and validation of the methods have not been performed.

Three of the studies[3-5] measured total circulating DNA by various methods. Fournie et al[5] reported increased circulating DNA levels in 36% of 68 cases, and in none of the 26 controls. Sozzi et al[5] quantitated circulating plasma DNA using DNA Dipstick (Invitrogen, Carlsbad, CA) and reported elevated levels in 45% of 84 cases and 0% of 43 controls using an optimal cutoff value. The receiver operator characteristic value was 0.84. In this issue's report, Sozzi et al report elevated circulating DNA levels, determined through the use of real-time quantitative PCR of the human telomerase reverse transcriptase (*hTERT*) gene, in 69% of 100 cases and 2% of 100 controls with a receiver operator characteristic value of 0.94.[3] This series is the largest reported, both in number of cases and controls. This report also has the best sensitivity and specificity for detecting cases among all the series reported. Using a different method of assessing *hTERT* DNA, Chen et al[6] reported elevated levels in four of 16 breast cancer patients and in none of the 23 controls. Although this study evaluated breast cancer patients as opposed to lung cancer patients, it is unclear why the levels of *hTERT* gene expression were so much lower. Quantitative PCR techniques have also been used to access other genes, including *ST4, hnRNPB1*, and *Her2/neu*.[7] Elevated expression levels of these genes were reported in 43%, 78%, and 39% of cases, respectively, in a small series. Although *hnRNPB1* levels were assessed in only 18 patients, the 78% rate was the highest of all the series presented in Table 1.

Methylation-specific PCR techniques have been used to quantify the methylation of the promoter regions of a number of oncogenes.[8-11] Four studies[8-11] used this technology to assess the frequency of abnormalities in the plasma of lung cancer cases and controls. Usadel et al[8] detected methylation of adenomatous polyposis coli (APC) in 95 (96%) of 99 cancers, primarily lung cancers, of which 47% had detectable amounts of methylated APC promoter DNA in plasma or serum. In contrast, no methylated APC promoter DNA was detected in serum samples from 50 healthy controls. However, other authors have reported far lower frequencies of methylated APC promoter DNA in primary lung cancers. Ramirez et al[9] used similar techniques to assess promoter methylation of the *TMS-1, RASSF1A*, and *DAPK* genes and reported abnormalities in 34% to 40% of 50 lung cancer cases (Table 1). Bearzatto et al[10] used methylation-specific PCR to look for methylation of the *p16* promoter in 35

Table 1. Summary of Studies Evaluating Serum Plasma Markers in Lung Cancer Patients and Controls

Reference	Marker	No. of Lung Cancer Cases	Positive Cases		No. of Controls	% Positive
			No.	%		
Fournie et al[4]	Total DNA	68	36	53	26	0
Sozzi et al[5]	Circ DNA	84	45	54	43	0
Sozzi et al[5]	QPCR *hTERT*	100	69	69	100	2
Chen et al[6]	QPCR *hTERT*	16*	4	25	23	0
Kopecki et al[7]	5T4mRNA	14	6	43	25	12
	RTPCRhnRNPB1	18	14	78	25	0
	Her2	18	7	39	25	0
Usadel et al[8]	Methylation APC	89	42	47	50	0
Ramirez et al[9]	Methylation TMS1	50	17	34	0	
	RASSF1	50	17	34	0	
	DAPK	50	20	40	0	
Bearzatto et al[10]	Methylation *p16*	35	12	34	15	
Esteller et al[11]	Methylation *p16, DAPK,* GSTpi, O6MGMT	22	11	50	11	0
Sozzi et al[12]	SMSA (2 markers)	87	35	40	14	0
Bruhn et al[13]	SMSA (3 markers)	43	14	33	10	0
Cuda et al[14]	SMSA (3 markers)	28	17	61	31	0
Chen et al[15]	SMSA (3 markers)	21	15	71	0	
Gonzalez et al[16]	SMSA (4 markers)	35	25	71	0	
Bearzatto et al[10]	SMSA (5 markers)	34	11	32	0	
Sozzi et al[5]	SMSA (5 markers)	38	9	24	43	0
Ramirez et al[9]	K-ras mutation	50	12	24	0	
Bearzatto et al[10]	K-ras mutation	35	0	0		
Total		969	433	45	403	1.2

Abbreviations: *DAPK,* death-associated protein kinase; TMS1, target of methylation inducing silencing; SMSA, serum microsatellite alterations; *hTERT,* human telomerase catalytic component; O6MGMT, O6 methylguanine methyl-transferase.

*In this study, 76.5% had at least one marker in gene. For breast cancer cases, all tumors were *hTERT* positive.

patients. Methylation was found in 34%. Esteller et al[11] combined methylation studies of four oncogenes and found that one or more were detectable in the plasma of 11 (50%) of 22 patients but in none of 11 controls.

Loss of heterozygosity and the presence of allele shifts indicating genomic instability have been studied in at least seven reports.[4,10,12-16] All series used two to five markers to increase the percentage of abnormal findings. Sozzi et al conducted allele shift analysis of D21S1245 with LOH of the *FHIT* locus, and found microsatellite alteration in 35 (40%) of 87 plasma samples.[12] Bruhn et al,[13] Cuda et al,[14] and Chen et al[15] each used three markers and reported microsatellite alterations in 33% of 43 cases, 61% of 28 cases, and 71% of 21 cases, respectively. These microsatellite alterations were not found in 41 controls. Gonzalez et al[16] used four markers and found that a higher percent were abnormal (71% of 35 patients). However, Bearzatto et al[10] and Sozzi et al[5] used five markers and found alterations in only 32% and 24% of cases, respectively. There was no uniformity of the selection of the markers used in these studies.

Two studies looked for *K-ras* mutations in serum or plasma. Ramirez et al[9] reported finding mutations in 12 of 50 cases, while Bearzatto et al[10] found no mutations among 35 cases.

Given the diversity of findings in these studies, where do we go from here? The results from the study of Sozzi et al in this issue of the *Journal of Clinical Oncology* are certainly provocative, with a sensitivity of 78% and a specificity of 95%, at a cutoff of 15 ng/mL; however, several steps are necessary before such tests are ready for "prime time." Within the studies presented in Table 1, there are many inconsistencies. For example, assays for *hTERT* varied in positivity from 25% to 69%, methylations of APC in the literature varies from 0% to more than 90%, *K-ras* mutations varied from 0% to 24%, and microsatellite alterations also varied considerably. These differences likely reflect variations in the manner of which the blood specimens were collected and handled, and variations in the methods by which the DNA/RNA assay were conducted. Thus, validation of collection and assay methods in different laboratories is critical. The cases and controls in these studies were not well matched. Pulmonary disease and changes in all epithelia induced by tobacco carcinogens should produce serum alterations. Thus, larger, better-matched control series are needed. A large, nested case-control series would be of considerable value.

Spiral computed tomography scanning is being assessed as another potential screening method for lung cancer in the National Lung Screening Trials (NLST) in the United States and in several trials in Europe. Serial blood samples are being collected in several, but not all of these trials (an unfortunate cost-cutting measure by the National Cancer Institute). When one of these DNA or RNA tests becomes validated by consistent results in several labs and confirmed in a large, matched control series, it will be important to study the samples being collected in the NLST and other spiral computed tomography trials to determine whether these serum analyses can help identify subjects at risk for lung cancer, compared with those with benign lesions. Serial analyses will be important in

this regard. Ultimately, a prospective mortality reduction study like the National Cancer Institute's Prostate, Lung, Colorectal, and Ovarian screening trial will be necessary to validate the use of these markers to reduce lung cancer mortality.

Finally, there is the issue of using these assays to observe patients, to predict their clinical outcomes. In the study of Sozzi et al,[5] 35 cancer patients had a second analysis 3 to 15 months after surgery. Median DNA concentrations fell from 24.5 ng/mL to 8.4 ng/mL ($P < .001$) after surgery. However, DNA concentrations fell in four of the five subjects who relapsed, and rose in three of the 28 subjects without relapse. Follow-up, however, is short, and additional time and subjects will be necessary to further evaluate changing levels as a prognostic tool.

In summary, tests for DNA or RNA alterations in plasma have great potential for early detection and follow-up. This study by Sozzi et al is a step forward in developing such a test. However, for lung cancer, much needs to be done in validation, and much larger series must be completed before these tests are ready for prime time.

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AUTHOR'S DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author indicated no potential conflicts of interest.

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